From the Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

MECHANISTIC STUDIES OF A NOVEL INHIBITOR OF THE UBIQUITIN-PROTEASOME SYSTEM

Ellin-Kristina Hillert

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MECHANISTIC STUDIES OF A NOVEL INHIBITOR OF THE UBIQUITIN-PROTEASOME SYSTEM

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Ellin-Kristina Hillert

Principal Supervisor:
Pādraig B. D'Arcy, PhD
Linköping University
Department of Medical and Health Sciences

Co-supervisor:
Professor Stig Linder
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Professor Andrew D. Westwell
Cardiff University
Dean of Research and Innovation
College of Biomedical and Life Sciences
School of Pharmacy and Pharmaceutical Sciences

Examination Board:
Tina Dalianis
Karolinska Institutet
Department of Oncology-Pathology

John Inge Johnsen
Karolinska Institute
Department of Women's and Children's Health

Peter Konradsson
Linköping University
Department of Physics, Chemistry and Biology

“One of the symptoms of an approaching nervous breakdown
is the belief that one’s work is terribly important."
- Bertrand Russell

Für Anne und Roger
Die besten Eltern die man sich wünschen kann

Abstract

The ubiquitin-proteasome system (UPS) not only maintains cellular proteostasis, but plays an essential role in multiple vital cellular processes, including survival and growth. Proteasomal inhibitors, such as bortezomib, have been shown to have effective anti-cancer properties, verifying the UPS as a viable drug target in cancer treatment. Yet, these compounds have encountered problems regarding toxicity, and inevitably development of resistance. The search for alternative targets within the UPS has revealed the 19S regulatory particle-associated deubiquitinases USP14 and UCHL5. Their inhibition blocks the deubiquitinating activity necessary for protein degradation by the proteasome. This has been shown to have cytotoxic effects in a range of cancer cells lines, as well as inhibiting tumor growth in several *in vivo* models. The small molecule inhibitor b-AP15 and its optimized lead VLX1570 were first discovered and characterized by the Linder research group at Karolinska Institute. Though thought to be highly promiscuous due to its α,β- unsaturated ketone motif, b-AP15 was demonstrated to selectively bind and inhibit the proteasomal deubiquitinases USP14 and UCHL5, with preferential binding to USP14. Inhibition of USP14 by b-AP15 results in a strong proteotoxic stress characterized by elevated levels of poly-ubiquitin, activation of the ER stress response, and oxidative stress, followed by apoptosis. We show here that the mechanism of b-AP15-induced apoptosis is characteristic of proteasome inhibition, but significantly differs from the effects of catalytic proteasome inhibitors. The results of b-AP15 treatment manifest as: severe proteotoxicity, mitochondrial damage without mitophagy induction, and lack of cytoprotective aggresome formation. Available evidence supports that the cellular response to b-AP15 is primarily dependent on USP14.

Additionally, we use a drug screen of compounds that share a reactive unsaturated ketone motif with b-AP15, to show their potential pharmacological applications, relative selectivity for USP14, and ability to inhibit the UPS.

This thesis describes in detail the proteotoxic effects induced by b-AP15 and its derivative VLX1570, and shows that despite its potential reactivity, b-AP15 selectively targets USP14. Similarly reactive compounds are shown to also display selectivity for the 19S deubiquitinases, indicating a potential for phamacological application in cancer therapy.

List of Publications

I. Xin Wang, Magdalena Mazurkiewicz, **Ellin-Kristina Hillert**, Maria Hägg Olofsson, Stefan Pierrou, Per Hillertz, Joachim Gullbo, Karthik Selvaraju, Aneel Paulus, Sharoon Akhtar, Felicitas Bossler, Asher Chanan Khan, Stig Linder, and Padraig D'Arcy
The proteasome deubiquitinase inhibitor VLX1570 shows selectivity for ubiquitin-specific protease-14 and induces apoptosis of multiple myeloma cells
Scientific Reports 2016; 6:269–279.

II. Xiaonan Zhang, Paola Pellegrini, Amir Ata Saei, **Ellin-Kristina Hillert**, Magdalena Mazurkiewicz, Maria Hägg Olofsson, Roman A Zubarev, Padraig B D'Arcy and Stig Linder
The deubiquitinase inhibitor b-AP15 induces strong proteotoxic stress and mitochondrial damage
Biochemical Phamacology 2018; 156:291–301.

III. **Ellin-Kristina Hillert**, Slavica Brnjic, Xiaonan Zhang, Magdalena Mazurkiewicz, Karthik Selvaraju, Arjan Mofers, Amir Ata Saei, Roman Zubarev, Stig Linder and Padraig D'Arcy
Proteasome inhibitor b-AP15 induces enhanced proteotoxicity by inhibiting cytoprotective aggresome formation
Cancer Letters 2019; 448:70–83

IV. Karthik Selvaraju, Arjan Mofers, Paola Pellegrini, Johannes Salomonsson, Alexandra Ahlner, Vivian Morad, **Ellin-Kristina Hillert**, Belen Espinosa, Elias S.J. Arnér, Lasse Jensen, Jonas Malmström, Maria V. Turkina, Padraig D'Arcy, Michael A. Walters, Maria Sunnerhagen, and Stig Linder
Cytotoxic unsaturated electrophilic compounds commonly target the ubiquitin proteasome system
Manuscript submitted for publication.*Scientific Reports*, under revision

V. **Ellin-Kristina Hillert**, Karthik Selvaraju, Arjan Mofers, Johannes Gubat, Stig Linder and Padraig D'Arcy, **Studies on the specificity of the deubiquitinase inhibitor b-AP15**
Manuscript

The articles will be referred to in the text by their Roman numerals, and are reproduced in full at the end of the thesis.

Related publications

- Magdalena Mazurkiewicz, **Ellin-Kristina Hillert**, Xin Wang, Paola Pellegrini, Maria Hägg Olofsson, Karthik Selvaraju, Padraig D'Arcy, and Stig Linder
  Acute lymphoblastic leukemia cells are sensitive to disturbances in protein homeostasis induced by proteasome deubiquitinase inhibition
  Oncotarget 2017; 8(13):21115–21127.

- Xin Wang, Magdalena Mazurkiewicz, **Ellin-Kristina Hillert**, Maria Hägg Olofsson, Stefan Pierrou, Per Hillertz, Joachim Gullbo, Karthik Selvaraju, Aneel Paulus, Sharoon Akhtar, Felicitas Bossler, Asher Chanan Khan, Stig Linder, and Padraig D'Arcy
  Corrigendum: The proteasome deubiquitinase inhibitor VLX1570 shows selectivity for ubiquitin-specific protease-14 and induces apoptosis of multiple myeloma cells
  Scientific Reports 2016; 6:30667.

Contents

List of Abbreviations

ATF6	Activating Transcription Factor 6
ATP	Adenosine Triphosphate
BRCA1	Breast Cancer Type 1 Susceptibility Protein
CdPT	Cadmium pyrithione
CHIP	C-terminal of Hsp70 Interacting Protein (E3 Ligase)
CP	Core Particle of the Proteasome
CuPT	Copper pyrithione
DUB	Deubiquitinase
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin Ligase
E6	HPV oncoprotein E6
E6-AP	Oncoprotein E6 associated protein
eIF2α	Eukaryotic initiation factor 2 α
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
GTP	Guanosine Triphosphate
HECT	Homology to E6-AP C terminal - E3 ligase family
HPV	Human Papilloma Virus
Hsp	Heat-shock protein
IAP	Inhibitor of Apoptosis protein
IRE1	Inositol-requiring enzyme 1
JAB1	c-Jun activation domain-binding protein-1
JAMM	JAB1-MPN-MOV34 motif
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
MDM2	Mouse double minute 2 homolog - E3 ligase
NFκB	Nuclear Factor kappa B
Nrf2	NF-E2-related factor 2 - transcription factor
OCR	oxygen consumption rate
P-SII	Partially selective Isopeptidase Inhibitor
p53	Tumor suppressor protein 53

PAINS	Pan assay interference compounds
PERK	Protein Kinase RNA-like endoplasmic reticulum kinase
PDB	Protein DataBank
PI3K-III	class III phosphatidylinositol 3-kinase complex
PINK1	PTEN-induced putative kinase 1
POH1	Rpn11 deubiquitinase
PGPH	Peptidyl-glutamyl hydrolysing
RIPA	Radioimmunoprecipitation assay buffer
RBR	RING between RING - E3 ligase family
RING	Really interesting new gene - E3 Ligase family
ROS	Reactive Oxygen Species
RP	Regulatory Particle of the Proteasome
Rpn	Regulatory particle non-ATPase
Rpt	Regulatory Particle Triple A ATPase
SAHA	Suberoylanilide hydroxamic acid/Vorinostat
SDS	Sodium Docecylsulfate
SQSTM1	sequestrome 1 / p62
TDP-43	TAR DNA-binding protein-43
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TrxR	thioredoxin reductase
TUBE	tandem ubiquitin-binding entities assay
Ub	Ubiquitin
UBA	Ubiquitin-associated domain
UBL	Ubiquitin-like domain
UCHL5	Ubiquitin carboxy-terminal hydrolase L5
UPR	Unfolded protein response
USP14	Ubiquitin-specific protease 14
UPS	Ubiquitin-Proteasome System
VCP	Valosin-containing protein - p97 AAA-ATPase
VHL	Von Hippel–Lindau tumor suppressor
YDR	Tyr-Asp-Arg motif
ZnPT	Zinc pyrithione

Chapter 1

Introduction

> *"Medicine is not only a science; it is also an art ... it deals with the very processes of life, which must be understood before they may be guided."*
>
> —Paracelsus

As Paracelsus reminds us, understanding the mechanism behind the action of a drug is an essential component of medical practice and research.

Especially in cancer research, a frequent practice it to report that a certain compound induces cell death, without further investigating why exactly it is doing so. Unsurprisingly, "death" is really not a very informative result, and it is certainly not enough to claim that a compound would make an effective cancer treatment. In order to understand how we can use a novel compound, we must always strive to understand how and why it works.

The focus of this thesis is understanding the mechanism and effects of b-AP15 - a novel inhibitor of the ubiquitin proteasome system - which targets the deubiquitinase USP14. By extension, this thesis also discusses the role of USP14 in proteasomal degradation, which has been a point of some controversy in the field of drug research over the past decade. This thesis therefore has two main focal points:

Firstly, this thesis deals with the cellular effects of b-AP15 on cancer cell survival (Paper I), mitochondrial function & damage (Paper II), and intracellular transport & aggresome formation (Paper III).

Secondly, this thesis focuses on the chemical and biological basis for the effects of b-AP15, and several of its derivatives. It describes in detail the rationale behind a drug-screen of compounds sharing a common enone motif with b-AP15 (Paper IV), and the dependence of b-AP15's cellular effects on the proteasomal deubiquitinase USP14 (Paper V).

Chapter 2

Background

2.1 The Ubiquitin-Proteasome System

The UPS is the major pathway of protein degradation in eukaryotic cells. It consists of a 2.5MDa catalytic complex and its associated ubiquitinating pathway - the ubiquitin system - which flags protein substrates for degradation. The proteasome itself consists of a catalytic core particle (CP, 20S) and one or two associated regulatory particles (RP, 19S), which together make up the 26S proteasome complex.[6, 7]

Originally thought to only be a recycling pathway for damaged proteins, it was not until the 1980s that the importance of this degradation machinery became known, revealing that protein stability is primarily determined by their degradation. Up until then, the maintenance of proteostasis was primarily attributed to translation, transcription and innate stability of proteins [8, 9, 10], and it was thought that degradation was primarily managed by lysosomes [11, 12, 13]. However, it was then observed that lysosome inhibition or absence does not prevent protein turnover, revealing that the UPS plays a central role in maintaining proteostasis, degrading as much as 80% of cellular protein [14, 15]. At the same time, the importance of the small protein ubiquitin in proteasomal degradation was uncovered. It has been shown that ubiquitin is an essential component of the degradation machinery, and that protein turnover hinges entirely on ATP-dependent linkage of ubiquitin to target proteins [16, 17, 18]. The discovery of this ubiquitin-dependent proteasomal degradation significantly advanced the understanding of cellular proteostasis, and lead to A. Ciechanover, A. Hershko and I. Rose being awarded the 2004 Nobel Prize in Chemistry.

Proteins targeted by the UPS include cell cycle regulators, receptors, signaling molecules, tumor suppressors and oncogenes[19, 20, 21, 22, 23, 24]. The proteasome is therefore tightly coupled to essentially all cellular processes, including the cell cycle, growth, signal processing, endocytosis and cell death [25, 26]. As such, its structure, assembly and function are stringently regulated by various mechanisms. Its central role makes the UPS a promising target not only in cancer therapy, but also for the treatment of neurodegenerative disorders, immunological dysfunction, cystic fibrosis and muscle wasting disorders [27, 28, 29, 30, 31].

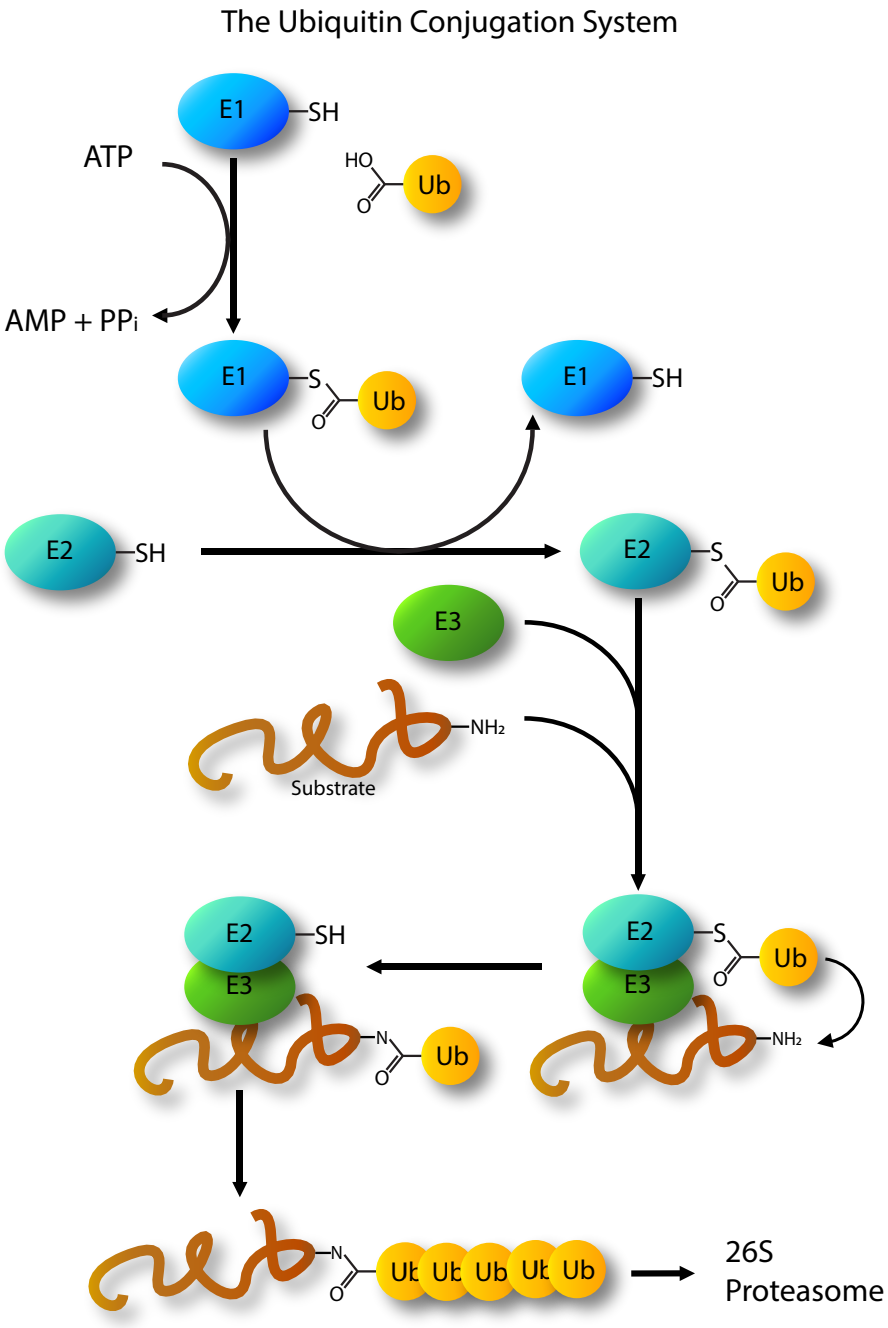


Figure 2.1: The Ubiquitin Conjugation system consisting of E1, E2 and E3 enzymes that work in sequence to link single ubiquitin moieties to substrate proteins in an ATP-dependent manner.

2.2 Ubiquitination and Regulation

Upstream of the 26S proteasome lies the Ubiquitin System. Its function is to recognize 26S protein substrates, and to tag them with the 76-amino-acid protein ubiquitin (Figure 2.1).

Ubiquitin is a highly abundant and conserved protein [32, 33]. The ubiquitin-tag targets the substrate to the proteasome for degradation, but can serve other functions as well. Ubiquitination requires the action of three different types of enzymes. First, the E1 (Ubiquitin Activating) Enzyme binds free ubiquitin and activates its C-terminal glycine residue (G76) in an ATP-dependent manner, creating ubiquitin adenylate. This glycine residue protrudes somewhat from the bulk of the protein, and is the sole known location at which covalent linkages to ubiquitin occur [34, 33]. The active glycine residue then binds to a cysteine on E1 [35, 36]. Secondly, the activated ubiquitin is transferred to a cysteine residue on the E2 (Ubiquitin Conjugating) enzyme. From there ubiquitina is finally transferred to an E3 enzyme (Ubiquitin Ligase) that will catalyze the linkage of the ubiquitin C-terminal glycine to the ε-amino group of a lysine on the target protein via an amide isopeptide linkage. There are over 600 distinct E3 ligases, which are classed into three subfamilies, depending on their structure and the mechanism by which they transfer ubiquitin to their substrate [37]. The most prolific family of E3 ligases are likely the RING (Really interesting new gene) finger ligases, which share the RING finger motif and simultaneously bind the E2-bound ubiquitin and the substrate to catalyze a direct ubiquitin transfer [38, 39]. The other two families are the HECT (homology to E6-AP C-terminus) E3 ligases, and RBR (RING between RING) E3 ligases. Both catalyze a sequential ubiquitin transfer by first accepting ubiquitin from the E2 enzyme, and transferring it onto a substrate in a second reaction [40, 41].

Only a single E1 enzyme exists. It can bind to all existing E2s, and transfer the activated ubiquitin for transient linkage. With multiple E2s, and several large families of E3 enzymes, specificity of the resulting ubiquitin tag increases with every step of the ubiquitination process [31]. Each E2 will only interact with a few select E3s, and each E3 has a high specificity for a particular substrate [42, 43]. This allows the Ubiquitin system to be tighly and specifically regulated.

Mono-ubiquitination plays an important role in cell signaling and regulating protein function [44, 45, 46], however, to target proteins for degradation, longer chains of ubiquitin moieties are required. To create these, the ubiquitination process is repeated, linking the next ubiquitin to a lysine on the first ubiquitin moiety resulting in poly-ubiquitin chains. There are at least 8 different subtypes of poly-ubiquitin linkages [47]. The most commonly occurring linkages connect the C-terminal Gly76 residue to the next ubiquitin's Lys48 or Lys63 residues. While Lys63-linked chains are important in non-proteolytic signaling - specifically protein kinase activation and DNA damage - [48, 43], Lys48 poly-ubiquitin targets substrates to the proteasome for degradation [49, 50, 31, 27]. A chain of at least four linked ubiquitin moieties is necessary for substrate recognition by the 26S proteasome [51, 52].

2.3 The Proteasome

The proteasome is an early evolutionary concept, with a conserved structure that can be found from archaebacteria to eukaryotes. It is localized in both the nucleus and the cytoplasm [53, 54, 55], and its essential functions are stringently regulated on various levels. Figure 2.2 shows the structural design of the 26S proteasome.

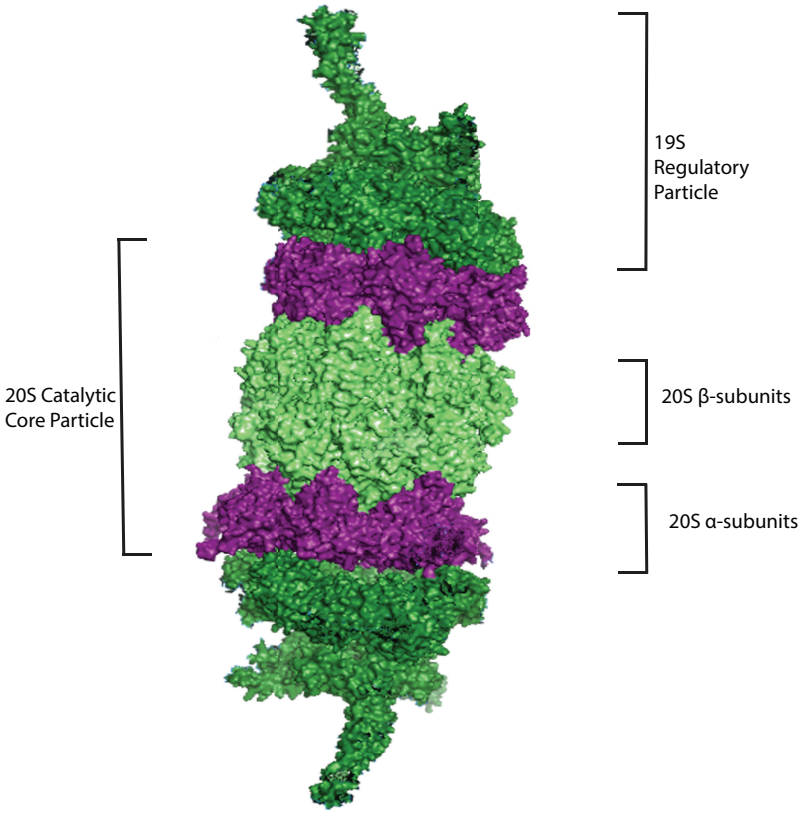


Figure 2.2: The structure of the 26S proteasome. The 19S RP is shown in dark green, the 20S α subunit rings are shown in purple, and the 20S β subunit rings are shown in light green (PDB:4CR2) [56, 57].

2.3.1 Structure and Function

The 26S proteasome is a large and complex multimeric structure, consisting of two distinct protein complexes: the 20S core particle (CP) and the 19S regulatory particle (RP) [58]. The 20S CP and the 19S RP carry out different functions that are essential to proteasomal degradation, and will be described in the following sections.

The 20S CP

The 20S CP is a large multimer, containing the catalytic activity of the proteasome. In eukaryotes it consists of 28 subunits. The subunits are arranged into 4 homoheptameric rings, which are

axially stacked, forming a barrel structure approximately 15nm long, 11nm wide and enclosing a cavity of 5nm diameter, with the catalytic activity contained inside. Eukaryotes have 14 distinct 20S subunits: $\alpha 1-7$ and $\beta 1-7$, and the 20S CP contains two copies of each. Each heptameric ring is made up exclusively of either α or β subunits. Two β rings form the center of the barrel, and two α rings form the ends, resulting in a $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ structure, with the N-termini of the subunits pointing inwards (Figure 2.2). All 20S subunits have a similar β-sandwich structure that is typical of N-terminal nucleophile-hydrolases [58, 59, 60]. Characteristically α subunits feature an extended N-terminal end, while β subunits are cleaved during proteasome assembly, to reveal an active threonine residue [61, 62]. However, only three of the β subunits are catalytically active: $\beta 1$, $\beta 2$ and $\beta 5$ are threonine proteases, carrying a single catalytic threonine (Thr1) which acts as a nucleophile in hydrolysis of peptide bonds. The N-terminal of Thr1 acts as the essential proton acceptor to achieve activation, while the surrounding residues Glu17, Lys33 and Asp166 are also required for catalysis [58, 63, 64]. The catalytic system itself is formed by the N-termini facing into the center of the barrel structure, with three active sites on each of the two β rings. The active sites have three major peptidase activities: peptidyl-glutamyl hydrolyzing (PGPH), trypsin-like and chymotrypsin-like, corresponding to $\beta 1$, $\beta 2$ and $\beta 5$ respectively [59, 65]. Each of the three different active sites has a specific cleavage site affinity. The $\beta 1$ PGPH (also referred to as caspase-like) activity cleaves primarily after acidic residues, $\beta 2$ cleaves after basic residues, and $\beta 5$ primarily cleaves after hydrophobic residues [58, 66].

It was once thought that the distance between, and specificity of these active sites would determine the size of the resulting cleaved peptides, like a "molecular ruler"[67, 68], with expected peptide sizes of 7 to 9 amino acids. However,it has been observed that the mammalian 26S proteasome produces a range of peptide lengths from 3 amino acids up to 30, with over 60 percent of peptides shorter than 8 amino acids and a mean length of 6 residues. Additionally, the bacterial proteasome has 14 active sites - all of which have chymotrypsin-like activity - yet it produces a similar spread of peptide products. The molecular ruler model therefore no longer holds up. Instead it has been proposed that substrates are simply degraded until they are small enough to diffuse out of the proteasome [69, 64]. In general it appears that the proteasome catalytic activity amounts to more than simply an integration of three different cleavage activities, and it has been shown that the proteasome can cleave its substrates at virtually any peptide bond, and that cleavage is influenced both by surrounding subunits, as well as residues other than the target cleavage site [70, 71, 67].

Arguably the main function of the structural organization of the 20S CP is to isolate and contain the proteolytic activity and prevent unwanted or uncontrolled degradation. Access to the catalytic chamber is therefore controlled, making substrate entry the rate limiting step in proteolysis. In eukaryotes the opening leading into the axial channel of the core particle is topologically closed by the tightly interwoven N-termini of the α subunits. This auto-inhibition prevents access for both folded and unfolded protein substrates [61]. The N-terminal of the $\alpha 3$ subunit in particular plays an essential role in stabilizing the closed conformation of the core particle. It carries the conserved YDR-motif containing an arginine residue (Arg9) that

has been shown to contact the N-termini of the surrounding α subunits [59, 61, 71]. To allow substrate access, a rearrangement of the α N-termini is necessary. This can either be achieved through binding to endogenous regulators or treatment with mild chemicals, such as sodium dodecylsulfate (SDS). Most commonly, the rearrangement and activation of the 20S CP is facilitated by binding to one or two 19S RPs [72, 73].

The 19S RP

The 19S RP is a 700kDa complex that associates with the 20S CP. It binds the ends of the barrel shaped structure formed by the 20S subunits and facilitates substrate entry and catalysis in an ATP-dependent manner (Figure 2.2). The 19S RP has at least 19 subunits and can be divided into two sub-structures, one proximal and one distal to the core particle, referred to as "base" and "lid" respectively. While the base is required for activation of the 20S proteolytic activity, the lid is necessary for degradation of ubiquitinated substrates. Both the base and lid contain at least 8 subunits. The base consists of a heterohexameric ring of AAA-ATPases (ATPase associated with different cellular activities): Rpt1-6 (Regulatory particle Triple A protein), as well as two organizing subunits; Rpn1 and 2 (Regulatory particle non-ATPase). Two established ubiquitin receptors - Rpn13 and Rpn10 - are bound to both Rpn1 and Rpn2 [74, 75, 76, 77, 78, 79]. The AAA-ATPases Rpt1-6 are Mg^{2+}-dependent and share a conserved 230-250 amino acid motif. They act in the ATP-dependent translocation of protein substrates into the 20S CP. Despite the strong similarity between them, they are not functionally redundant, and seem to carry different specificities for proteasome substrates [80, 81, 82]. The lid consists of one constitutive deubiquitinase (DUB) - Rpn11/POH1 - bound to the non-catalytic Rpn8 subunit, as well as 7 scaffolding proteins: Rpn3,Rpn5,Rpn6,Rpn7,Rpn9,Rpn12 and Rpn15. All but Rpn15 contain a conserved PCI scaffolding domain at the C terminal, which serves as the contact point between the subunits. This lends the lid a vaguely hand-shaped structure with Rpn3,7,6,5 and 9 as the 'fingers' and the Rpn11 DUB at the centre of the palm, surrounded by Rpn8, 9 and 5. The constitutive ubiquitin receptor, Rpn10, located in the base, carries a VWA globular domain that bridges Rpn11 and 9 [83]. In binding, the 19S RP modifies the conformation of the 20S α N termini, allowing for pore opening and substrate entry, and forming the 26S holoenzyme complex known as the proteasome.

2.4 Proteasomal degradation of substrates

Ubiquitination alone is not enough to target a substrate for degradation. In addition to carrying a poly-ubiquitin chain the target protein must also have an unstructured region, that will serve as the starting point for degradation. The Lys48 ubiquitin chain will bind the proteasome at one of its several ubiquitin receptors, either the pleckstrin-like domain of Rpn13, one of the two UIM (ubiquitin interacting motifs) of Rpn10, or the UBA domains found in the shuttle receptors. This places the unstructured region of the substrate within reach of the AAA-ATPase hexamer of the 19S base, where it can engage the ATPase motor. ATP hydrolysis leads to conformational

changes in Rpt1-6, that drive the translocation of the unfolded substrate through the pore into the 20S CP [84, 85]. Degradation of the substrate occurs progressively as the protein is unwound starting at the attachment point, while being translocated into the 20S chamber for proteolysis [86]. The ubiquitin tag is removed as part of this translocation by the proteasomal deubiquitinases. Multi-ubiquitin chains that have been removed en bloc from their protein substrate are degraded into monomers by other ubiquitin-specific peptidases in the cytosol, including Isopeptidase T [29, 87].

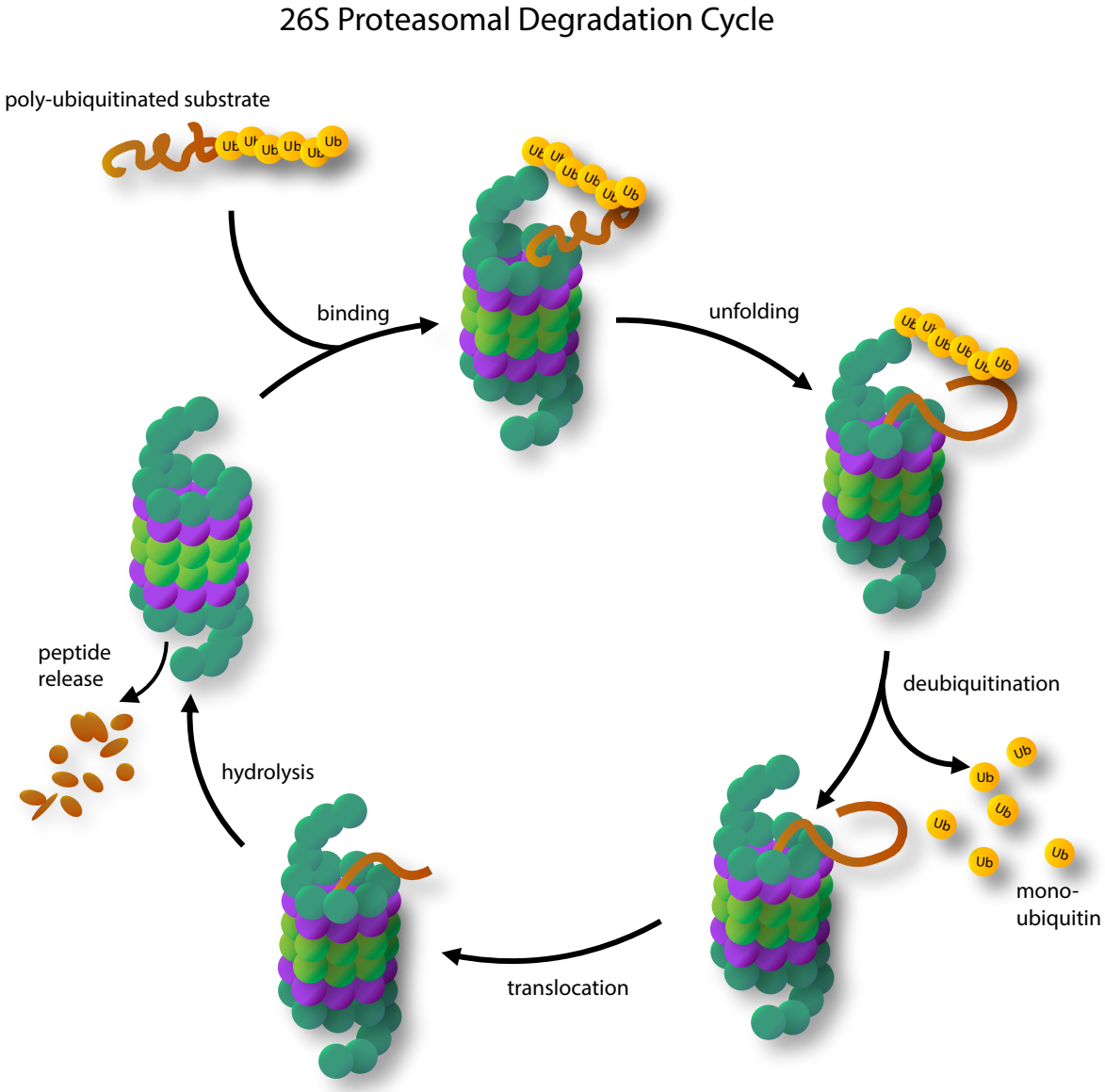


Figure 2.3: Steps of the 26S proteasomal degradation cycle.

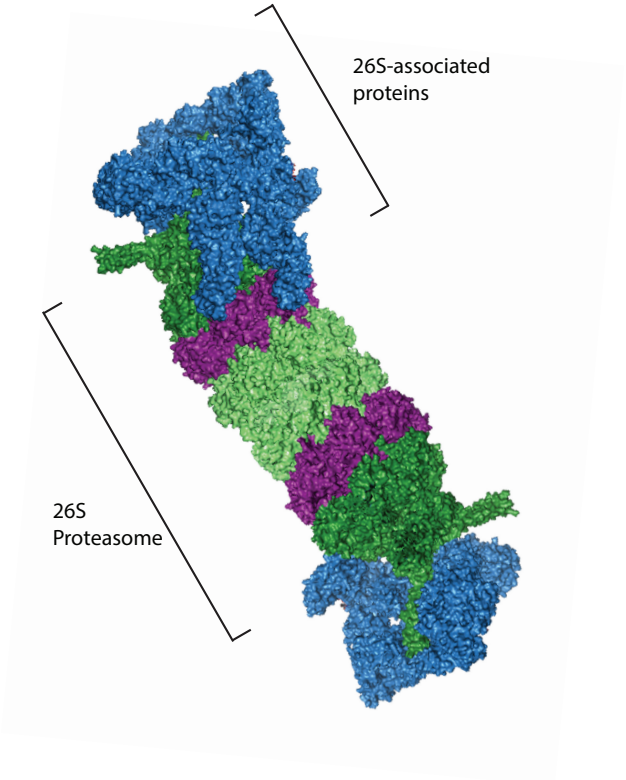


Figure 2.4: The location of 26S proteasome-associated proteins, shown in blue, which bind the 19S RP and include shuttle receptors and deubiquitinases.(PDB:5GJQ)

2.5 Proteasomal Deubiquitinases

Other proteins also associate with the proteasome, and modify degradation events (Figure 2.4). This includes the additional shuttle receptors for ubiquitin: Rad23, Ddi1 and Dsk2 (ubiquilins in mammals). These shuttle receptors have some redundancy with the constitutive ubiquitin receptors, but show higher specificity for certain subsets of degradation substrates. All three contact 19S Rpn1 via their N-terminal ubiquitin-like (UBL) domains and bind poly-ubiquitinated substrates with their C-terminal ubiquitin-associated (UBA) domains [88, 83, 76, 29].

Other 19S associated proteins include the deubiquitinases that carry out the essential removal of poly-ubiquitin chains from the 26S substrates. Removal of the chains replenishes the cell's pool of free ubiquitin and allows the protein to be unfolded and translocated into the proteasome. This chain removal is carried out by proteasomal deubiquitinases that specifically hydrolyse ester, thiol ester or amide bonds to the Gly76 residue of ubiquitin. The 19S-associated DUBs consist of the constitutive JAB1-MPN-MOV34 (JAMM) metalloproteinase Rpn11/POH1, as well as two, more loosely associated, DUBs, UCHL5/uch2/ and USP14/ubp6 (human/yeast nomenclature). POH1 is a metalloprotease that is thought to be the main source of deubiquitinating activity on the proteasome, while the role of the other two proteasomal DUBS is not as well understood. While POH1 is an endo-deubiquitinase that shows degradation-coupled activity, ubiquitin cleavage by UCHL5 and USP14 appears to be independent of degradation. Both are thought to be exo-deubiquitinases, that cleave the ubiquitin chain form the distal

end, rather than removing the entirety of it from the substrate protein [89, 90]. This function may serve in ubiquitin chain editing, but it is uncertain whether these two DUBs function in promoting or preventing degradation. This ambiguity has been the source of some controversy in the field of DUB research [89, 91].

2.5.1 Rpn11/POH1

POH1 cleaves the ubiquitin chains at the proximal end in a Zn^{2+}-dependent manner. It is the only constitutive DUB on the proteasome, and forms an integral subunit of the 19S RP. It is thought to be regulated in part through conformational changes induced by RP binding to the 20S CP [92, 83, 93], but can also be influenced by the activity of the other two DUBs [94]. It is currently believed that for a ubiquitin chain to trigger degradation of its bound protein it has to contact both Rpn10 and Rpn13, spanning a distance of $\sim$ 90Å. This means that a ubiquitin chain must contain at least 4 ubiquitin moieties in order to successfully target a protein to the proteasome. The binding of the chain to the proteasomal ubiquitin receptors then orients the substrate protein in a way that facilitates cleavage of the ubiquitin chain by POH1, as well as unfolding and translocation. Cleavage of the ubiquitin chain is essential for degradation to proceed, and loss of DUB activity has been shown to be lethal. At the same time, deubiquitination by POH1 will only occur in a degradation coupled manner, that is, cleavage happens once the substrate is committed to degradation[89, 95, 96].

2.5.2 UCHL5

UCHL5 is present in stoichiometric amounts in the 19S subunit, where its C-terminal associates with that of Rpn13. Rpn13 is in turn associated with the base subunit Rpn2 via its N-terminal. Binding to Rpn13 has been shown to increase UCHL5 activity, and appears to stabilize the ubiquitin binding site of the deubiquitinase. UCHL5 is specific for the distal end of poly-ubiquitin chains, preferring Lys48-linked ubiquitin [97, 98, 99, 100, 101]. It interacts with multiple residues of the most distal ubiquitin, including Lys48, and may stabilize a salt bridge between Lys48 and Glu51 within ubiquitin. This interaction limits iUCHL5 binding to the most distal ubiquitin only, explaining its exo-specificity [102].

2.5.3 USP14

USP14 is the third 19S-associated deubiquitinase, and the main drug target discussed in this thesis. Like UCHL5, USP14 is associated with the 19S RP in stoichiometric amounts, and is the most abundant proteasome-associated protein [103]. It binds the Rpn1 subunit in the base of the RP, via its UBL domain, with a higher affinity than any of the ubiquilin shuttle receptors, yet it also dissociated easily from the 19S RP. Binding of USP14 to Rpn1 has been shown to increase its deubiquitinating activity up to 300-fold, while free USP14 appears to have very little catalytic activity [104, 91, 105].

The structure of the USP14 catalytic USP domain has been reported as a "right hand", with sub-domains referred to as "fingers", "palm" and "thumb", resembling several other known

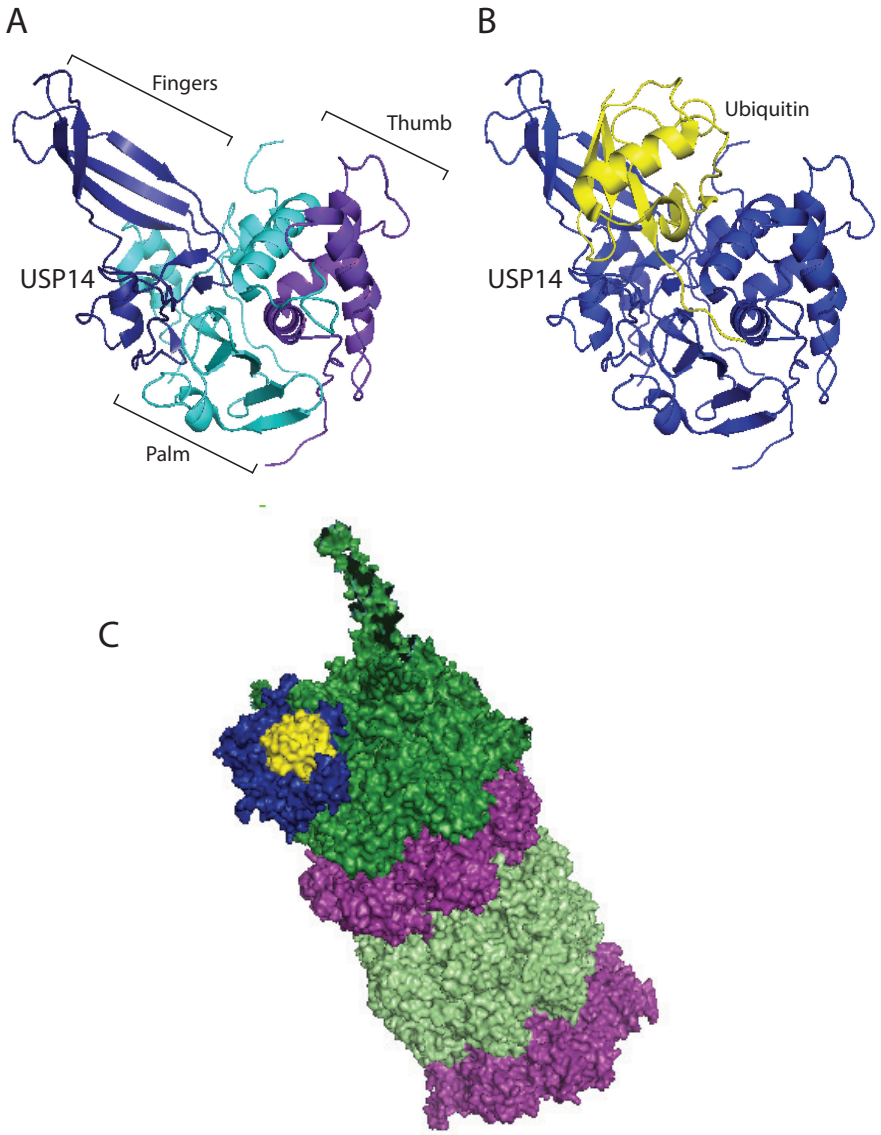


Figure 2.5: The structure and location of the proteasomal deubiquitinase USP14 A) General USP14 structure, with the sub-domains (fingers, palm and thumb) indicated in blue, cyan and purple respectively. B) Ubiquitin aldehyde (yellow) bound to USP14 (blue) in the ubiquitin binding pocket between the fingers and thumb sub-domains. C) The location of USP14 (blue) on the 26S proteasome, including ubiquitin bound to USP14 (yellow) (PDB:2AYO and 5GJQ)

DUBs (Figure 2.5). The ubiquitin binding site is located between the fingers and the thumb. The USP14 active site is located between the fingers and the palm. Unlike several other DUBs, its catalytic triad is already properly aligned prior to substrate binding. However, access to the catalytic triad is blocked by two loops (BL1 and BL2). Binding of ubiquitin leads to a conformational change in USP14, which removes several residues that otherwise sterically restrict access to the catalytic triad. This rearrangement allows the ubiquitin C-terminal to reach the catalytic site via a cleft connecting the ubiquitin binding pocket to the catalytic triad (Figure 2.6) [106]. Upon binding to Rpn1 via its Ubl domain, the USP domain remains somewhat flexible. Association of ubiquitin with the catalytic domain fixes the domain location in close proximity to the Rpt1 ATPase, bridging the gap between Rpt1 and POH1. This places the USP catalytic triad in close proximity to both the ATPase active site, and the POH1 DUB, and suggests a high level of crosstalk between these subunits in order to coordinate proteasome activation and substrate degradation [103, 107, 94]. The USP14 catalytic triad itself (Figure 2.6) consists of a catalytic cystein residue (Cys114), histidine (His435) and aspartic acid (Asp451). The imidazole ring of the catalytic histidine is thought to form a hydrogen bond with the thiol group of Cys114, an interaction that may be stabilized by Asp451.

Poly-ubiquitin binding by USP14 has been shown to trigger gate opening in the 19S RP, if it occurs in concert with ATP binding to the 19S RP AAA-ATPases [108]. If USP14 binds poly-ubiquitin that is associated with a partially unfolded protein substrate, it promotes ATPase activation, and therefore translocation of the substrate into the 20S catalytic chamber for degradation [109]. Simultaneously, USP14 has been shown to stabilize the substrate-bound conformation of the proteasome, while in return the same active proteasome conformation increases USP14 deubiquitinase activity [94].

The actual role USP14 plays in proteasomal degradation is, however, somewhat controversial. While some have claimed that its primary function is to delay or prevent proteasomal degradation, others believe that USP14, like POH1 is an instrumental component in enabling degradation by deubiquitinating proteasomal substrates prior to them entering the catalytic chamber [110]. There are a multitude of publications in support of either side, which has lead to USP14 being proposed as a drug target in cancer treatment (inhibiting the proteasome by USP14 inhibition) [111, 112, 113], as well as a target in the treatment of neurological disorders such as Huntington's disease or Alzheimers (promoting clearance of protein aggregates via the UPS by inhibiting USP14) [114, 115].

In an attempt to unify both sides, some recent publications have suggested that USP14 serves a dual function. According to these proposals, non-ubiquitin-bound USP14 suppresses the basal peptidase activity of the proteasome, while ubiquitin-bound USP14 increases proteasomal ATPase activity, substrate translocation and degradation. Moreover, blockage of the 20S catalytic activity, and build-up of poly-ubiquitinated protein substrates quickly increases the association of USP14 with the proteasome, while absence of poly-ubiquitin will cause rapid dissociation [107, 116]. Similarly, inhibition of USP14 using ubiquitin aldehyde, or other ubiquitin-based active site inhibitors, leads to increased binding of USP14 to the proteasome [104]. It has been suggested that this mechanism serves to encourage proteasomal selectivity for ubiquitinated

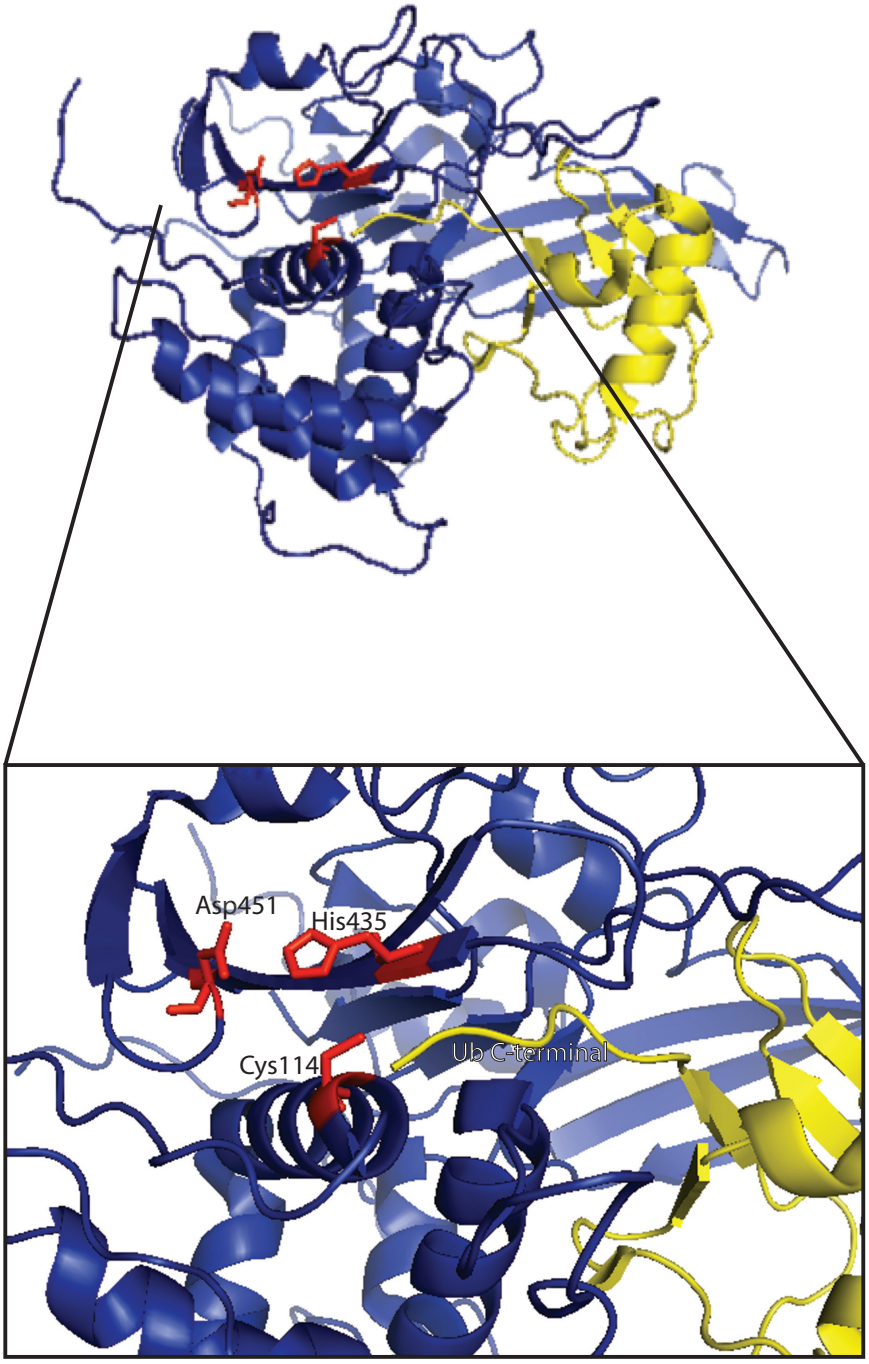


Figure 2.6: The catalytic triad (red) of USP14 (blue) consisting of Cys114, His435 and Asp451. The ubiquitin (yellow) C-terminal has access to the catalytic triad for cleavage (PDB:2AYO).

proteins, if such proteins are present [107, 116].

The role of USP14, and its use as a drug target will be discussed further later in this thesis. However, a considerable body of evidence- including our own data- indicates that removal or inhibition of USP14 is a trigger for proteotoxic stress.

2.6 Proteotoxic stress and Proteostasis

Proteasome overload, dysfunction, or inhibition has detrimental effects on cell health and survival. Maintenance of proteostasis is therefore a key process in any cell. Previous research has shown that it may be especially important in cancer cells that produce high amounts of unfolded or mutated protein, which are therefore described as having a non-oncogenic addiction to proteasomal degradation [117, 118]. Proteasome inhibition has been shown to severely upset cellular proteostasis, leading to apoptosis via an increase in ER stress, accumulation of reactive oxygen species (ROS) and mitochondrial damage [119, 120, 121].

ROS, including hydrogen peroxide, as well as superoxide and hydroxyl radicals are a byproduct of oxidative metabolism in mitochondria. In healthy cells, ROS levels are maintained by an antioxidant system that prevents oxidative damage to proteins and organelles, while oxidized proteins are degraded by the proteasome [122, 123]. Proteasome inhibition has been shown to induce both ER stress and mitochondrial damage, both of which cause an increase in cellular ROS levels, compounding the existing proteotoxic stress the cell is experiencing. Since the direct cytotoxic effects of ROS are somewhat debatable [124], the role of ROS in apoptosis via proteasome inhibition is uncertain. However, it has been shown that proteasome inhibition leads to mitochondrial membrane depolarization [125], thereby triggering a mitochondria-dependent caspase cascade, culminating in apoptosis [126, 127]. Cells possess several partially redundant mechanisms to protect themselves against proteotoxic stress and ensure their continued survival. These mechanisms include autophagy, the ER stress response, and aggregate formation, and are summarized in Figure 2.7.

2.6.1 ER stress

The endoplasmic reticulum (ER) is the first step in the cellular secretory pathway that manages synthesis, modification and delivery of proteins within the cell [128, 129]. There is evidence of extensive crosstalk between the UPS and the ER [130, 131], and proteasome inhibition is a known inducer of ER stress [132, 120, 133].

Nascent unfolded proteins, are translocated into the ER for processing. This involves the folding or re-folding of protein chains, as well as disulfide bond formation. Folding is managed by various ER molecular chaperones, prior to shipping of the folded protein to the Golgi, or other intracellular compartments via vesicles [134]. The protein folding pathway is highly conserved from bacteria to mammals [129]. ER stress describes the event when the folding capacity of the ER becomes overloaded, i.e. the volume of unfolded protein within the ER becomes too high. This can occur due to a variety of cellular stresses, including thermal stress, mutations, hypoxia

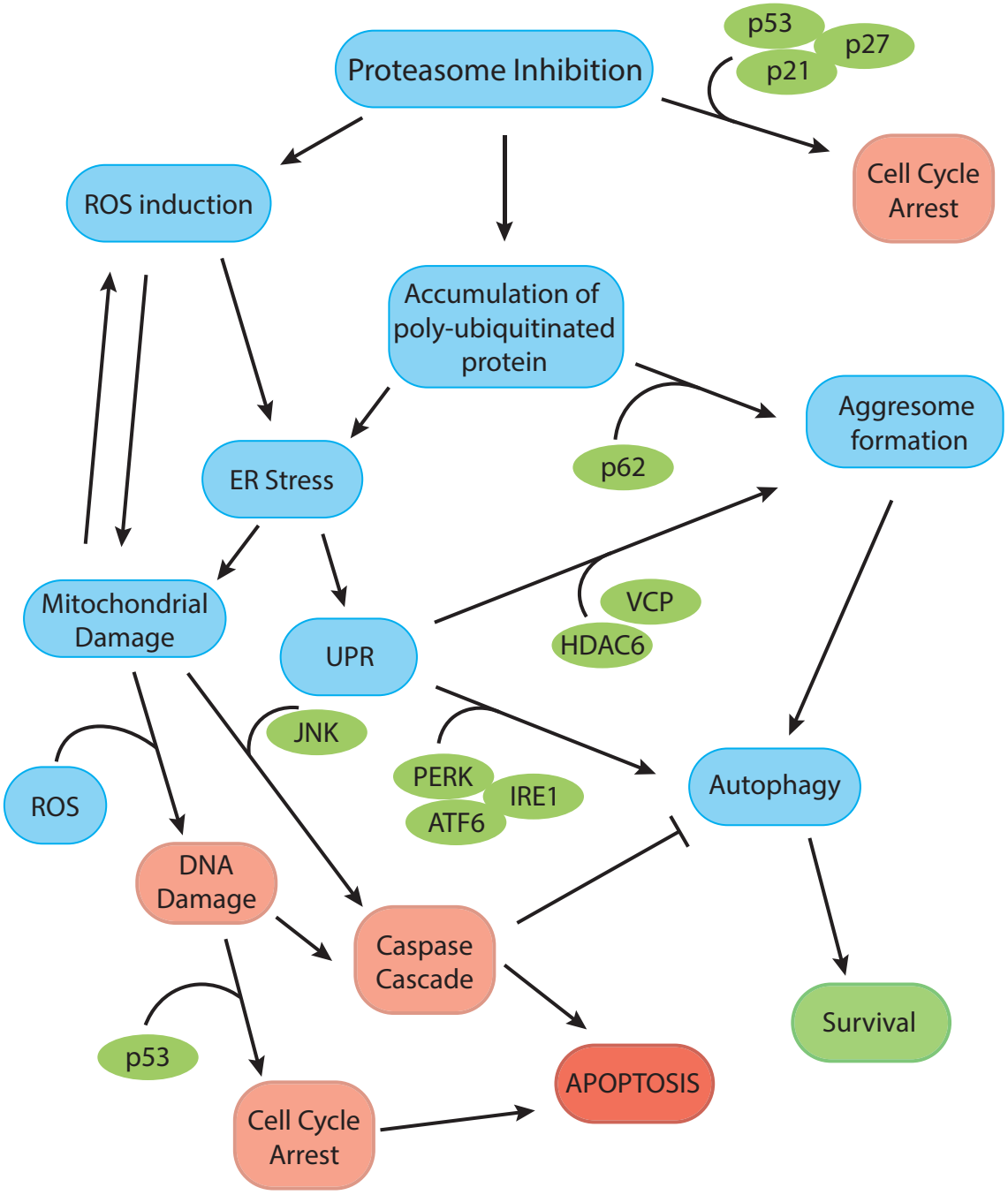


Figure 2.7: Effects and cellular response of proteasome inhibition

or infection [135, 132]. Proteasome inhibition will increase the concentration of unfolded protein outside the ER, yet triggers the ER stress response. This may be due to increased calcium levels as a result of proteasome inhibition, or due to the interaction of unfolded protein with cytosolic chaperones [136, 137, 138, 139].

In the event of ER stress, the Unfolded Protein Response (UPR) is activated. Three transmembrane proteins manage the UPR in mammals: the kinases IRE1 and PERK, and the transcription factor ATF6 [140, 133]. These proteins are located in the ER membrane, with a stress-sensing domain facing the ER lumen, and a functional domain facing the cytosol. The ER-internal domain is bound to BiP, a heat-shock protein 70 (Hsp70). BiP has a high affinity for hydrophobic patches on unfolded proteins. In the presence of unfolded proteins in the ER lumen it will dissociate from the domains of the three transmembrane proteins, allowing them to activate and trigger the UPR [140, 141, 142]. The UPR has two main functions: reducing the folding demand in the ER, and increasing the ER folding capacity [135]. However, prolonged activation of the UPR will trigger cell death [143, 144], thus the UPR will activate both pro-survival and pro-death functions [145].

Both IRE1 and ATF6 modulate increased transcription of chaperones to increase the ER's protein folding capacity [146, 147]. In order to reduce ER folding demand the UPR will downregulate protein translation, and upregulate ER-associated degradation (ERAD). Translation is downregulated via PERK-mediated phosphorylation of eIF2α. As a component of the translation pre-initiation complex eIF2α must bind GTP in order to facilitate recognition of the mRNA start codon. Phosphorylation at Ser51 by PERK prevents nucleotide exchange, thereby preventing translation initiation [148, 149, 150, 151, 152]. PERK also phosphorylates Nrf2, as part of its second pro-survival function, in order to increase glutathione levels - an antioxidant that will combat the increase in reactive oxygen species (ROS), associated with ER stress [153].

ERAD is primarily activated by IRE1-mediated splicing of XBP1 mRNA - a highly active transcription factor that is controlled by ATF6 [147, 154]. Like the rest of the UPR, ERAD depends on the activity of ER chaperones, that bind the unfolded protein in the ER lumen [142]. ERAD will effect the removal and extraction of unfolded protein from the ER, and deliver them to the proteasome for degradation [155, 156] in a process called retrotranslocation or dislocation. This reduces the ER's folding load. ERAD substrates are recognized and bound by Hsp chaperones (Hsp40, Hsp70 or Hsp90), which will then recruit the E3 ligase CHIP. How exactly the protein substrates are removed from the ER is currently not fully understood. A putative protein channel termed the dislocon is thought to be responsible for the transport, which seems to be both ATP- and ubiquitin-dependent. Several E3 ligases are responsible for ubiquitinating the protein substrates as they emerge for the ER [157, 158, 159]. The AAA-APTPase p97/VCP has been reported as not only a main regulator of proteostasis [160], but also the primary linkage between ERAD and the UPS [161]. VCP is required for the extraction of unfolded protein from the ER, as well as other organelles including mitochondria. In the case of ERAD activation, p97/VCP is recruited to the ER membrane, where it associates with the ER-bound E3 ligase Hrd1. Hrd1 ubiquitinates emerging ERAD substrates and causes them to be targeted to the proteasome [162]. VCP then forms a complex with the ubiquitin-binding

proteins Np14 and Udf1 to bind the ubiquitinated ERAD substrates and deliver them to the proteasome, with the help of proteasome shuttle receptors such as Dsk2 and Rad23 [163, 156, 164].

2.6.2 Aggregate formation

The accumulation and aggregation of misfolded or damaged proteins is a common occurrence in any cell [165], and is tightly linked to ER-stress, proteasomal degradation and autophagy. Protein accumulation can be induced by changes in protein structure due to mutations, thermal stress and oxidative stress, as well as some types of viral infections, which will lead to ER stress, resulting in ERAD and increased cytosolic levels of unfolded protein [166, 167]. These proteins would normally be targeted to the proteasome for degradation and clearance [22, 6].

In the event of insufficient proteasome activity, such as in severe cases of ER stress, proteasome inhibition, or oxidative stress, poly-ubiquitinated unfolded proteins must be removed from the cytosol. To this end, these proteins are trafficked along the microtubule network to form a single large inclusion body at the microtubule-organizing center (MTOC) in close proximity to the nucleus [168, 165]. This inclusion body is referred to as an aggresome.

Aggresomes have been found to contain not only poly-ubiquitinated protein, but also several chaperones, especially HSPs, which are known to be upregulated in cells as a first line of defense against proteotoxic stress [169]. Aggresomes also contain components of the USP, such as proteasomal subunits, as well as various adapter proteins that act in the aggregation, segregation and the eventual autophagic clearance of aggresomes. The process of aggresome formation and clearance is fairly complex and not throughly understood. It involves interaction of the substrate proteins with several enzymes, including p97/VCP, SQSTM1/p62 and HDAC6, as well as motor proteins and the cytoskeleton [170, 171].

The AAA-ATPase VCP/p97 facilitates the extraction of unfolded proteins from the ER during ERAD, and then further controls the transport of these proteins towards the proteasome [162]. In the event of a proteasomal defect, cells may upregulate the histone deacetylase HDAC6. This unique deacetylase contains a C-terminal zinc-finger motif that allows it to bind to ubiquitin chains with high affinity [171, 172]. HDAC6 also contains a dynein-binding domain that allows it to link unfolded protein to dynein motors on the cytoskeleton for transport to the aggresome at the MTOC [169]. Aggresome formation is therefore dependent on cytoskeletal integrity.

While the temporary accumulation of poly-ubiquitinated protein in the aggresome removes potentially harmful aggregates from the cytoplasm [173, 174], this is only a temporary solution to proteotoxic stress. Aggresome formation is thought to facilitate the autophagic clearance of toxic protein aggregates, by creating a single locus for the recruitment of autophagic machinery [175]. There is a strong body of evidence suggesting that SQSTM1/p62 is the main link between aggresome formation and autophagic clearance of aggregates [176, 177]. SQSTM1/p62 is a stress induced scaffold protein that serves as the main adapter protein in autophagy [178]. Additionally p62 appears to actively promote aggregation of poly-ubiquitinated proteins in the cytosol by forming p62 filaments when bound to poly-ubiquitin chains [179], thus linking the

poly-ubiquitinated proteins into larger clusters.

2.6.3 Autophagy

Autophagy is the second major pathway of protein degradation in cells after the UPS. While the UPS is mostly responsible for the dynamic degradation of short-lived and soluble proteins, as well as ERAD substrates, autophagy primarily targets non-soluble, long lived proteins, as well as protein aggregates and entire organelles for degradation [180]. Autophagy is a a lysosome-dependent pathway, in which degradation is carried out by a large specialize organelle, called the autophagosome [181]. Autophagic degradation can be divided into two sub-categories : Unselective autophagy, and selective autophagy. Unselective autophagy has the primary function of cellular nutrient recycling and maintaining homeostasis [182], which protects the cell from stress and disease [183]. Selective autophagy targets specific proteins.

Initially thought to be an entirely non-specific pathway for degradation, autophagy has now been shown to be capable of specifically targeting substrates, as well as having significant crosstalk with the UPS [184, 185]. Like UPS-dependent degradation, selective autophagy is determined by ubiquitin labeling of the substrate - primarily unfolded protein. The ubiquitinated proteins are then targeted by sequestrome 1 (p62/SQSTM1) [186] and other autophagy-related proteins, such as HDAC6 [187] and HSPs [188]. These proteins recruit autophagic machinery to the site of the unfolded protein, and promote clearance [189, 188]. The process of autophagy consists of four distinct steps: induction, cargo recognition and selection, autophagosome formation, and fusion of the autophagosome with the lysosome. Inside the lysosome the cargo is then degraded and its components are released into the cytosol [190].

The autophagic response can be initiated via ERAD, in response to ER stress. It also serves as a redundancy mechanism in case of proteasome dysfunction or inhibition [133, 191]. Autophagic clearance is the primary mechanism of aggresome removal after proteasome inhibition, thus promoting cell survival [192]. The sequestrome 1 protein p62/SQSTM1 is the primary link between UPS-mediated degradation and autophagy. Recent publications have shown that p62 is an essential component of the aggresome-formation mechanism that clears misfolded protein from the cytosol in the event of proteasome overload or dysfunction [176, 179, 193]. It also serves as a ubiquitin-binding adapter protein in autophagy. This allows the aggregates to then function as signaling nodes to promote autophagic clearance, modulated by p62 [178, 194].

Additonally, p62 is an important component of the Keap1-Nrf2 signaling pathway. Under normal conditions p62 is poly-ubiquitinated by the Keap1-Rbx1 E3 ligase complex, targeting it for autophagic degradation. In the event of proteotoxic stress it becomes phosphorylated at its Ser349 residue- This allows p62 it to interfere in the ubiquitination of the transcription factor Nrf2 by Keap1. Once stabilized, Nrf2 translocates to the nucleus, where it activates transcription of autophagy-associated genes, antioxidants and p62 itself [178, 195].

Put simply: Cells can employ the above responses in an attempt to correct conditions of proteotoxic stress. The ER stress response will increase the ER folding potential, while targeting

excess unfolded protein to the proteasome. VCP will extract proteins from the ER and functions in trafficking them to the proteasome. In the event of proteasome dysfunction, it will pass the unfolded protein substrates on to HDAC6 for trafficking to the aggresome, where p62 is involved in recruiting autophagic machinery for aggregate clearance. These processes serve to protect the cell from cytosolic protein toxicity. However, none of these responses are a sustainable solution, and prolonged proteotoxic stress will eventually force the cell to undergo apoptosis.

2.6.4 Cell Death

Proteotoxicity-induced apoptosis can be triggered in a variety of ways. It has been shown that apoptosis induced by proteasome inhibitors is primarily effected via ER stress or mitochondrial damage [196, 143, 197], as well as the increased levels of cytosolic pro-apoptotic proteins, such as p53, as well as $p27^{Kip1}$ and $p21^{Cip1}$, which will cause cell cycle arrest in the G2/M phase [198]. Increased levels of p53 will lead to upregulation of pro-apoptotic proteins, including Bax, and will lead to cell cycle arrest and apoptosis [199].

Prolonged ER stress will result in ER-activated autophagy (ERAA) and apoptosis by upregulating the pro-apoptotic transcription factor ATF4 (activating transcription factor 4), which in turn activates transcription of CHOP (C/EBP homologus protein) and GADD34 (Growth Arrest and DNA-damage inducible gene 34). CHOP induces transcription of TRAIL-R1/DR4 and TRAIL-R2/DR5 [200, 201, 202], which mediate caspase-8-dependent apoptosis [203, 204]. It also causes the translocation of the pro-apoptotic protein Bax to the mitochondria, triggering mitochondria-dependent apoptosis via caspase 3 [205, 197]. GADD34 recruits protein phosphatase 1 (PP1) to the ER, where it will dephosphorylate eIF2α, reversing the translational repression that was part of the initial ER stress response [206]. IRE1 activation by ER stress will lead to the activation of JNK (c-Jun N-terminal Kinase), as well as caspase 4 [207]. JNK phosphorylates the anti-apoptotic protein Bcl-2, interfering with its binding and inhibition of the pro-autophagy Beclin 1. Active Beclin 1 is a component of the PI3K-III (class III phosphatidylinositol 3-kinase) complex, which is responsible for the recruitment of autophagy machinery and membrane components [208, 209], thus triggering autophagy. Additionally active JNK can translocate to mitochondria, where it will stimulate the release of cytochrome c from the inner mitochondrial membrane. This process is dependent on pro-apoptotic Bid and Bax, and initiates the activation of caspase-9-dependent caspase cascade, culminating in apoptosis [210].

A delay in autophagic clearance of aggresomes has been shown to lead to DNA damage and p53-mediated cell cycle arrest [211]. It has been found that agents which disrupt aggresome formation, such has HDAC6 inhibitors, synergize with proteasome inhibitors in the induction of cytotoxicity in cancer cells [212, 213], consistent with the idea that aggresome formation has a cytoprotective function [214, 215, 216, 217, 218, 219].

Strategies that inhibit aggresome formation, to enhance cytotoxicity induced by established proteasome inhibitors, such as bortezomib, have been generating increased interest in recent years [213, 220]. Given the dependence of many cancer cells on efficient proteasomal degradation and ROS management, targeting these processes is a popular strategy in cancer drug

development. Compounds that trigger ER stress are being investigated for potential adjuvant use with proteasome inhibition. Inducing or increasing proteotoxic stress, or interfering with the compensatory mechanism cells can use to alleviate proteotoxic stress, as a means to rapidly induce commitment to apoptosis, are also an appealing potential method of targeting cancer cells [221, 222, 223].

2.7 The UPS in Cancer and Disease

Due to its vital functions in a multitude of essential cellular processes, UPS malfunction has been implicated in the pathogenesis of a broad rage of diseases, including cystic fibrosis, immune dysfunction and muscle wasting disorders [28]. The most prevalent of the diseases involving the UPS are neurodegenerative disorders and cancer. The UPS involvement can be explained by either a loss of function of degradation, or a gain of function.

Loss of function, leading to stabilization and accumulation of proteasome substrates, is of primary importance in neurodegenerative disorders, such as Alzheimer's and Huntington's disease. Gain of function, where substrates are degraded at an accelerated rate, is relevant in cancer development, as will be described in detail [9, 24].

2.7.1 The UPS in Neurodegeneration

Several neurodegenerative diseases including Huntington's Disease, Alzheimer Disease, and Parkinson's, involve abnormal protein aggregates and inclusion bodies. Theses aggregates have been shown to include both poly-ubiquitin and proteasomes, implicating a dysfunction of protein degradation, where the substrates are labelled for degradation, but not removed [9, 224]. While proteasome impairment may be part of the underlying cause for neurodegenerative disorders, protein aggregates have in turn been shown to inhibit proteasome function. Amyloid β plaques may inhibit all three catalytic activities of the 20S CP. A clear link between the UPS and neurodegeneration has yet to be established, but work is under-way to employ enhancement of proteasomal degradation as a treatment strategy in a variety of disorders [50].

2.7.2 The UPS in Cancer

In cancer, UPS activity and dysfunction play a major role, which is not surprising considering how many cellular processes are regulated via protein degradation. The UPS and its regulation are especially important in cell cycle progression and cell death induction; processes that require the specific and well-timed removal of various active proteins [26]. Components of the UPS are therefore common targets of oncogenic mutations [25]. Frequent sites of these mutations are various E3 ubiquitin ligases or their components. Examples include the E3 component VHL, which is involved in degradation of hypoxia-induced transcription factors (HIFs). VHL is known to be a gatekeeper mutation in renal clear cell carcinoma, as its loss of function prevents HIF degradation, leading to a hypoxia response under normoxic conditions - inducing angiogenesis and promoting tumor growth [225, 226]. The breast-cancer type 1 susceptibility

protein BRCA1 is also known to carry E3 ligase activity [227]. While its exact function as an E3 ligase is not fully understood, it is known that mutations in the BRCA1 Zn^{2+}-binding region - which is responsible for its ubiquitin ligase activity - predispose to breast cancer development [228].

Various oncogenes and tumor suppressors are also degraded via the UPS. This includes the cell cycle regulators p53 and $p27^{Kip1}$. The p53 tumor suppressor is a main component of cellular stress response, and is responsible for inducing cell cycle arrest, senescence and apoptosis in response to DNA damage [229, 230, 231]. The cellular levels of p53 are regulated by the MDM2 E3 ligase, which targets p53 to the proteasome for degradation [232]. Amplification of MDM2 is commonly found in p53-wt cancers, where it reduces p53 levels and allows for unchecked cell cycle progression [233]. Several high-risk strains of of human papilloma virus (HPV) take advantage of this pathway. The viral HPV E6 protein will bind to the cellular E6-AP E3 ligase. The resulting dimer then ubiquitinates p53, targeting it to the proteasome [234, 235, 236]. The cyclin-dependent kinase inhibitor $p27^{Kip1}$ is also a proteasome substrate, and can be down-regulated by the UPS [237, 238, 239]. Down-regulation of $p27^{Kip1}$ occurs in various cancers, where it correlates with poor prognosis [240, 241, 242].

Other oncogenes targeted by the UPS include c-Myc, n-Myc, Jun, and cyclin E [21]. At the end of each mitotic cycle, cyclin-specific E3 ligases target cyclins for destruction by the proteasome [243]. Aberrant levels of cyclin E have been shown to correlate with poor prognosis and survival in several types of cancer, leading to tumor formation, chromosome instability and cell cycle dysregulation [244, 245, 246].

Various deubiquitinases have also been implicated in cancer initiation and progression. This includes both of the 19S-associated deubiquitinases, USP14 and UCHL5, which have been found to be overexpressed in a range of cancer types, and are associated with less favorable prognoses[247]. Inhibition of these two proteasomal DUBs has been shown to have cytotoxic effects in a range of malignancies [248, 249, 111].

Proteasome activity has been shown to be increased in many cancers. This may be due to higher levels of protein translation as a result of aberrant proliferation rates. Other contributors are elevated oxidative stress, production of mutant proteins, as well as frequently increased levels of exposure to cytokines and growth factors, experienced by cancer cells [118]. In fact, the reliance of many cancer cells on UPS-mediated protein degradation has in recent years been described as a type of non-oncogenic addiction [117]. Accordingly, proteasome inhibition has been shown to slow or block cancer growth and angiogenesis in a variety of cancer models [250]. Additionally UPS inhibition can stabilize the levels of tumor suppressors, including p53 and $p27^{Kip1}$, curbing unchecked cell division [251]. Its central role in essential cell processes and the common reliance of cancer cells on rapid protein degradation make the UPS an attractive therapeutic target in cancer therapy [9, 50, 252, 253].

2.8 Targeting the UPS in Cancer Treatment

The idea to target the UPS in the treatment of various disorders, and either enhance or inhibit its function, depending on the desired effect, has been under investigation for several years [254, 255]. Especially the development of UPS inhibitors, targeting an array of components of the ubiquitin-proteasome system, has been a focal point of extensive research in recent years. Additionally the development of proteasomal DUB inhibitors has generated increasing interest [256]. Potential targets within the UPS are abundant, and research has been done on a multitude of its components. The most promising targets include E3 ligases, the 20S catalytic core and the 19S regulatory particle.

2.8.1 Targeting the ubiquitin cascade

Inhibitors have been developed for each step of the ubiquitination pathway, targeting E1, E2 and E3 enzymes. Due to their high specificity and the almost 600 known E3 ligases, they may be the most promising, as they provide the most targeted option for intervention. Several E3 ligases have no conserved active site, making targeting challenging, especially of HECT type E3 ligases. The E3 inhibitors that are in development primarily target RING- type E3's, by preventing the association of their several subunits, or by preventing substrate binding [257]. Examples are RITA and MI-219, two compounds that target the MDM2/HDM2 (mouse/human) E3, which specifically ubiquitinates p53. Preventing HDM2-p53 association stabilizes p53, allowing for p53-mediated cell cycle arrest and apoptosis to proceed [258]. Another promising target in cancer therapy are the IAP (Inhibitors of Apoptosis) family, which contain several RING E3 ligases [259]. IAPs are frequently found to be upregulated in cancer, promoting cancer cell survival by inhibiting caspase activation and apoptosis. IAP inhibition with RITA or nutlin compounds allows the caspase cascade to proceed and leads to apoptosis [257, 260, 261].

Another method of inhibiting the ubiquinating machinery is by directly targeting ubiquitin chains, and preventing their association with the proteasome. Several compounds, termed ubistatins, can achieve this by binding the ubiqitin-ubiquitin interface of K48-linked poly-ubiquitin chains, preventing recognition of the chains by the protasomal ubiquitin receptors. This is a non-specific approach that prevents the degradation of a broad spectrum of cell cycle components and regulators, including cyclin B and p53, and leads to cell cycle arrest [257, 262, 263].

2.8.2 Targeting 20S

Targeting the 20S catalytic core particle inhibits total protein degradation in the cell. While this approach is broader than inhibition of specific E3 ligases, it has shown promising effects in the clinic. While the early generation 20S inhibitor MG132 is quite potent and of considerable use in *in vitro* experiments, it displayed instability *in vivo* [264]. The primary example of a 20S inhibitor with clinical application is the peptide boronate bortezomib, the first proteasome inhibitor to be approved by the FDA [265].

Bortezomib

The 20S inhibitor bortezomib (Velcade®/ PS-341) is a boronic acid dipeptide [266] that targets the catalytic $\beta 5$ subunits of the 20S CP, inhibiting the chymotrypsin activity in a reversible manner [267, 268]. It has a biological half-life of about 24h [269] and has been shown to stabilize proteasome substrates including p53 and $p27^{Kip1}$, as well as pro-apoptotic members of the Bcl-2 protein family, leading to apoptosis [270]. The structure of bortezomib is shown in Figure 2.8. The apoptotic effect of bortezomib has been primarily attributed to its induction of increased levels of the pro-apoptotic protein NOXA, as well as an inhibition of NFκB signaling [207], a response that is characteristic of proteasome inhibition [251]. This method of targeting the proteasome has been shown to be more effective in cancer types that display increased protein synthesis and ER stress. Examples include multiple myeloma as well as mantle cell lymphoma, which characteristically show amplified synthesis of proteins, especially immunoglobulins [271]. In clinical use, bortezomib elicited a good response in 48% of new patients, and a 35% response rate in patients with relapsed and refractory multiple myeloma [266]. However, resistance to bortezomib is a common occurrence, either through mutation or overexpression of the $\beta 5$ subunit [272, 273, 274] or protective mechanisms mediated by HSPs [275]. An additional resistance mechanism may involve the overexpression of mutant Bcl-2 protein. It has been shown that myeloma and lymphoma cells that express Bcl-2 mutants with a higher affinity for NOXA display resistance to the pro-apoptotic effects of increased NOXA levels. Further difficulties in the clinical use of bortezomib stem from dose-limiting toxicity issues and interactions with some natural compounds [207].

As the first FDA-approved inhibitor of the UPS, bortezomib has however inspired further research into alternative methods of proteasome inhibition, leading to the discovery of a whole range of other compounds that are currently under development.

Next generation 20S inhibitors

The second generation 20S inhibitor Carfilzomib (Kyprolis®) is an epoxyketone, based originally on the drug epoxomicin [278] (Figure 2.8). It irreversibly targets the same binding site on the $\beta 5$ subunit as bortezomib, and demonstrated overall higher specificity and activity than bortezomib [279, 280]. Carfilzomib has been FDA approved as a treatment in relapsed and refractory multiple myeloma [281]. However, it has been shown that resistance to bortezomib also decreases the efficacy of carfilzomib, suggesting that this second-generation inhibitor is vulnerable to acquired resistance by the same mechanism as bortezomib [282].

Other second generation 20S inhibitors include the epoxyketone oprozomib, as well as the reversible boronic acid inhibitors delanzomib and ixazomib. All of these inhibitors are primarily intended for use in multiple myeloma, and all are potent cytotoxic compounds [280]. Much like bortezomib, promising anti-neoplastic effects were primarily observed in hematological malignancies, while results for 20S inhibitors in solid tumors have been mostly discouraging [207].

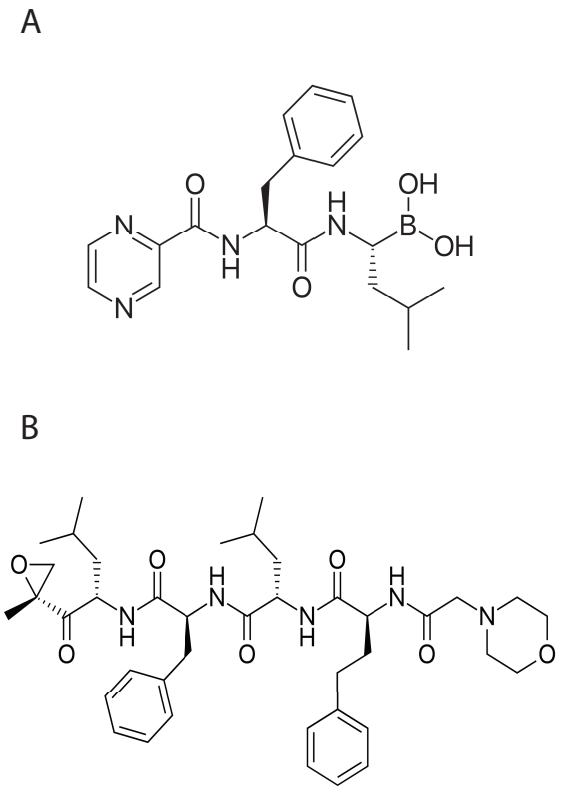


Figure 2.8: The 20S inhibitors A) Bortezomib and B) Carfilzomib [276, 277]

2.8.3 Targeting 19S

The most promising targets located on the 19S RP are the ubiquitin receptors within the lid, and the 19S-associated deubiquitinases. Ubistatins (A and B) have been successfully used to inhibit ubiquitin chain binding to the proteasome ubiquitin receptors. These compounds bind to the Ub-Ub linkage in Lys48 ubiquitin chains, preventing recognition by the receptors [262]. The RA-190 compound is reported to target the constituitive 19S ubiquitin receptor Rpn13, preventing ubiquitin binding and substrate degradation [283], which has shown anti-cancer potency in multiple myeloma cell models [284, 285].

The proteasomal deubiquitinases USP14 and UCHL5 can be inhibited by several compounds, including the small molecule inhibitor b-AP15, causing accumulation of proteasomal substrates and poly-ubiquitin, eventually leading to cell death [111]. The following sections will focus on targeting these two proteasomal deubiquitinases in cancer treatment in more detail.

2.9 Targeting 19S Deubiquitinases in Cancer Treatment

Both USP14 and UCHL5 have been reported to show increased expression in a variety of cancers [286], where they are frequently associated with worse prognoses and survival [112, 113]. Thus, the 19S DUBs have garnered increasing interest as potential drug targets in cancer therapy [111, 256]. Inhibition of USP14 has been shown to be an effective method of intervention in several types of cancer, including squamous cell carcinoma [287], multiple myeloma [288],

melanoma [289], Waldenström macroglobulinaemia [290, 291], and colorectal cancer [249].

Targeting DUBs has been technically simpler than targeting E3 ligases, partially due to their lower diversity, and the conserved cysteine residues found in the majority of deubiquitinases. USP14 and UCHL5 both contain conserved active site cysteines. The reactivity of cysteines lends itself to effective targeting using electrophilic compounds. POH1, however, is a metalloprotease, which is can be targeted by zinc-chelators such as thiolutin [292], but generally is not sensitive to the same compounds that will bind to the other two proteasomal DUBs. This makes it possible to develop compounds that target USP14 and UCHL5 only, without risking the general cytotoxic effects of POH1 inhibition.

The use of electrophilic compounds can be problematic due to general reactivity within the cell, that can result in proteasome-independent cytotoxicity, and may prevent achieving high specificity for particular DUBs [293, 113]. Yet, recently significant advances regarding DUB targeting and function have been made. An array of compounds has been developed that target both USP14 and UCHL5 deubiquitinases with varying degrees of specificity, some of which show a high degree of promise [113, 294, 256, 295, 296].

2.10 Deubiquitinase Inhibitors

Established proteasomal deubiquitinase inhibitors include an array of natural compounds as well as synthesized derivatives and novel molecules. The idea that an α,β-unsaturated ketone motif (or enone) is the essential component of certain types of DUB inhibitors has been established and explored over the course of the last two decades [296]. Unsaturated ketones are a common feature of many DUB inhibitors that have been developed, while others function by alternative mechanisms that frequently target the DUB catalytic cysteine.

2.10.1 Curcumin

The yellow plant extract curcumin (tumeric, Figure 2.9) has long been used in traditional medicine. Curcumin has validated anti-tumor effects, as well as known benefits for a wide range of other disorders. It is one of the most widely studied anti-tumorigenic compounds today [297, 298]. Curcumin's broad range of effects is most likely due to the inhibition of proteasomal activity, and the resulting accumulation of poly-ubiquitin, followed by cell death in sensitive cells. However, its exact mechanism of action is unknown. Its structure, featuring an α,β-unsaturated ketone group, may target reactive conserved cysteines found in DUBs [299], preventing proteasomal deubiquitination, and effectively blocking protein degradation. However, direct effects on the 20S CP have also been described. 19S DUB inhibition may therefore only be a contributing factor to the anti-neoplastic activity ascribed to curcumin.

Curcumin has been shown to be effective in cancer treatment, despite fairly poor bioavailability [300, 301, 302, 113]. Other natural compounds that likewise carry an α,β-unsaturated ketone have been shown to have broad range anti-DUB activity, and also target UCHL5 and USP14. This includes betulinic acid and gambogic acid, both also known in traditional medicine

[303, 304].

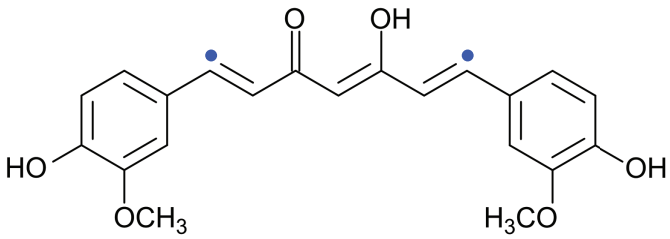


Figure 2.9: Chemical structure of curcumin, indicating the α,β-unsaturated ketone motifs in blue [305, 298]

2.10.2 AC17

The 4-arylidene curcumin analogue AC17 follows a similar mode of action, also carrying an α,β-unsaturated ketones, allowing it to target USP14 and UCHL5 irreversibly. This has been shown to lead to inhibition of protein degradation without blocking the 20S proteolytic activity (unlike curcumin, which also targets proteolysis). AC17 treatment results in accumulation of poly-ubiquitin, as well as inhibition of the NFκB pathway and stabilization of p53, cumulating in apoptosis [306]. More recently, several additional 4-arylidene curcumin analogues were reported as potential 19S DUB inhibitors, all of which feature the same α,β-unsaturated ketone motif [307].

2.10.3 WP1130

Another DUB inhibitor with reported anti-tumour activity is the small molecule WP1130, which has been shown to have broader inhibitory functions, targeting USP9x and USP5 along with USP14 and UCHL5. Similarly to the previously described compounds it results in apoptosis of malignant cells via poly-ubiquitin accumulation and increase of pro-apoptotic proteins [308] as well as activation of the unfolded protein response [309]. Research suggests that the anti-cancer effects of WP1130 are primarily due to its inhibition of USP9X, resulting in the proteasomal degradation of various anti-apoptotic proteins including c-Myc and Bcr/Abl [310, 311]. The unsaturated ketone motif can be found here too. However, WP1130 only carries a single one.

2.10.4 Auranofin

The gold-containing compound auranofin was originally FDA approved as a treatment for rheumatoid arthritis, and has since been repurposed for applications in immunosuppression, and cancer treatment [313]. Auranofin has been shown to induce cellular proteotoxicity as well as inhibition of USP14 and UCHL5, resulting in proteasome-blockage and toxicity to cancer cells. However, USP14 inhibition only occurs at concentrations exceeding IC_{50} [316]. It has been suggested that an intermediate form of the compound allows the chelated gold ion (Au+) to directly target the USP14 and UCHL5 catalytic triads [312].

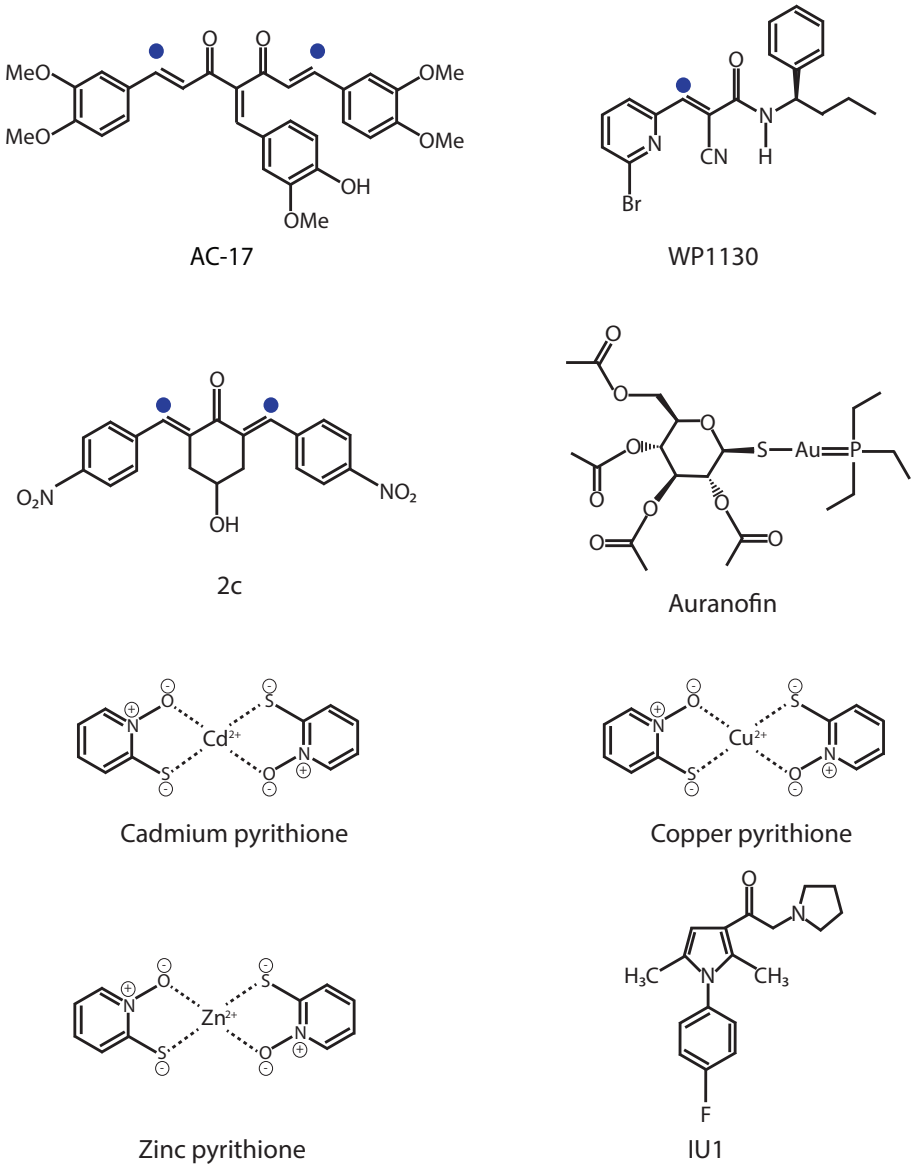


Figure 2.10: Chemical structures of a range of established 19S DUB inhibitors. If present, unsaturated ketones are indicated in blue [306, 308, 312, 313, 314, 315, 114]

2.10.5 Metal pyrithiones

Along with auranofin, several metal containing compounds have recently gained the spotlight as potential 19S DUB inhibitors. One focus has been on metal pyrithiones - chelating complexes of metal ions- such as copper pyrithione (CuPT), cadmium pyrithione (CdPT) and zinc pyrithione (ZnPT). Both CuPT and ZnPT have previously known properties as anti-fouling components in paint and anti-dandruff agent respectively [317, 318]. Recent research has revealed that these metal pyrithiones are potent UPS inhibitors that lead to the accumulation of poly-ubiquitin, and caspase-dependent cell death in cancer cell lines, primary tumor cells and xenograft models [314, 319, 318]. All three have been shown to target USP14 and UCHL5, leading to UPS inhibition. CuPT and CdPT also display 20S inhibitory properties at higher concentrations. As with auranofin, a potential docking mechanism suggests that a metal-containing intermediate form of the chelating complex interacts directly with the catalytic triad of USP14 and UCHL5 [318, 313].

2.10.6 G5/2c

The research and increased awareness of the phamacological properties of α,β-unsaturated ketones [296] was put to a targeted use in the development of the bis-arylidenecycloalkanone isopeptidase inhibitor G5 [320], and later its derivative 2c [321]. The 2c compound, tagged with biotin, was successfully used as bait to isolate possible targets of these types of molecules, which the group termed Partially Selective Isopetidase Inhibitors (P-SIIs) [315]. A pull-down screen revealed targeting of the DUBs USP1, USP18, USP14, UCHL5 and USP33. An additional mass spectrometry screen indicated targeting of several other proteins, some of which are also involved in cellular proteostasis.

2.10.7 IU1 and the USP14 controversy

The USP14-selective inhibitor IU1 was first described in 2010 by a group working on the premise that USP14 and its yeast homologue UBP6 inhibit proteasomal degradation [110, 114]. While in yeast the inhibitory effects of UBP6 were of an allosteric nature, it appeared that USP14-dependent proteasomal inhibition in humans was dependent on its catalytic activity. Following this reasoning, an inhibitor of USP14 catalytic activity would have potential applications in treatment of neurodegenerative diseases, where it could increase the removal of aberrant proteins. IU1 was the result of a drug-screen with the objective of finding such an inhibitor. It was reported to reversibly inhibit USP14 catalytic activity at an IC_{50} of 4-5μM, and the ability to increase degradation of tau and TDP-43 *in vitro* and in cells [114]. IU1 was not reported to induce apoptosis.

2.11 b-AP15 as a DUB inhibitor

Targeted inhibition of USP14 has been achieved with the DUB inhibitor b-AP15. This small molecule has been shown to specifically bind USP14 and UCHL5, although showing higher affinity for USP14. It has no visible activity in a panel of other DUBs, and specifically inhibits USP14 at pharmacological concentrations of 1μM, without inhibition of related deubiquitinases. Additionally, b-AP15 treatment did not decrease total cellular DUB activity [322, 323, 111].

2.11.1 b-AP15 structure

The b-AP15 compound also contains the commonly observed α,β-unsaturated ketone. This motif contains two Michael acceptors - electrophilic groups that are thought to target reactive cysteine residues, such as the catalytic cysteine in the active site of both USP14 and UCHL5. Despite being a covalent modification, b-AP15 inhibition has been shown to be reversible. However the commitment to apoptosis induced by b-AP15 is irreversible. This inhibitor has been shown to have anti-neoplastic effects in a variety of malignancies including acute lymphoblastic leukaemia, multiple myeloma, and Waldenström macroglobulinemia [323, 324, 291].

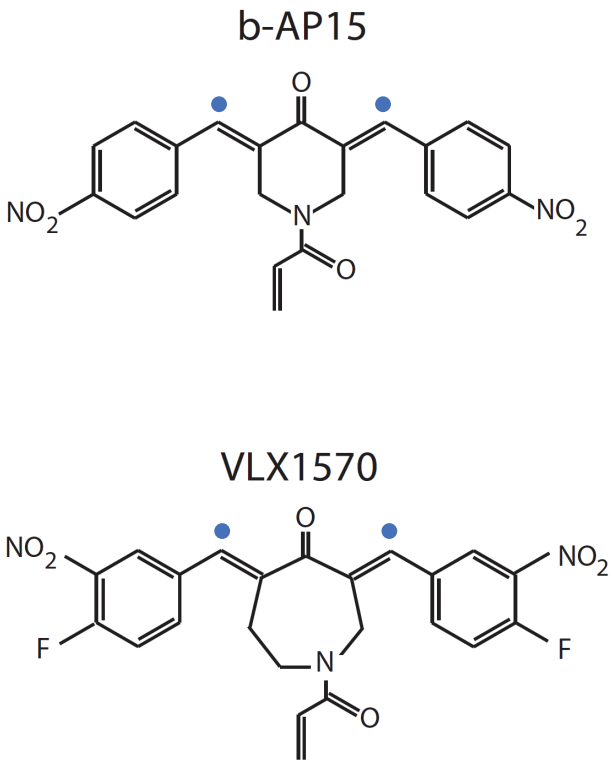


Figure 2.11: The structure of the small molecule inhibitor b-AP15 and its derivative VLX1570. The unsaturated ketones characteristic of these molecules are indicated in blue.

2.11.2 b-AP15-induced proteotoxicity

Treatment of cancer cells with b-AP15 induces the accumulation of poly-ubiquitin, as seen with other inhibitors of proteasomal DUBs as well as the 20S CP, however, apoptosis induced by b-AP15 is independent of both p53 and Bcl-2 status of the cells. Since both of those factors strongly influence bortezomib sensitivity, b-AP15 may have an advantage in terms of applicability. Additionally, b-AP15 cytotoxic effects in cancer cells were also observed in multiple myeloma cell lines resistant to bortezomib, highlighting its potential as a second-line treatment after relapse [111, 113].

In addition to poly-ubiquitin accumulation, b-AP15 has also been observed to induce oxidative stress and ER stress in cancer cells. This is not uncommon, as other proteasome inhibitors also induce oxidative stress, however, b-AP15 has also been confirmed as a thioredoxin reductase (TrxR) inhibitor[288, 325], which may cause it to induce higher levels of ROS than conventional proteasome inhibitors. A significant drawback to the clinical use of b-AP15 is its limited solubility, nevertheless b-AP15 has been observed to be enriched and retained in cancer cells [288].

2.11.3 b-AP15 derivatives

Due to the solubility problems of b-AP15, efforts were made to develop similar compounds, that would display increased solubility and specificity for USP14 [326]. All resulting derivative retained the α,β-unsaturated ketone motif, which is believed to be responsible for their pharmacological activity. The optimized lead VLX1570 displayed the lowest IC_{50} values in HCT116 cells, and was selected for further characterization in Paper I.

VLX1570

The optimized analogue VLX1570 (Figure 2.11), has shown increased solubility in aqueous solution, as well as high specificity for USP14 and UCHL5, where it acts as a competitive inhibitor [326]. VLX1570 was subsequently shown to induce apoptosis in multiple myeloma cells in a manner consistent with the effects of USP14 inhibition, exhibiting poly-ubiquitin accumulation, oxidative stress and eventual apoptosis (Paper I). The cellular response produced by VLX1570 closely resembles that of b-AP15, and is consistent with proteasome inhibition. Additionally, VLX1570 has been FDA approved as a treatment for relapsed and refractory multiple myeloma [327].

Novel enone-containing compounds

Following a similar rationale as the development of the G5/2c inhibitor development [320, 321, 315], Current efforts are going towards identifying other 19S DUB inhibitors from a panel of compounds that have one or more unsaturated ketones. The prevalence and popularity of several natural compounds with these α,β-unsaturated ketone motifs, suggests that despite their proclaimed reactivity, there may be potential clinical applications for them. A screen of

5000 compounds revealed a range of molecules that display general cytotoxicity, as well as several potential selective USP14 and UCHL5 inhibitors. Efforts to find additional promising compounds that target proteasomal deubiquitinases are described in detail in Paper IV.

Chapter 3

Aims of this Thesis

The overall aim of this thesis is to elucidate the mechanism of action of the novel 19S deubiquitinase inhibitor b-AP15 and its derivatives.
While it was repeatedly shown that b-AP15 targets the proteasomal deubiquitinase USP14, the mechanism by which this induces proteotoxic stress and apoptosis was poorly understood. The objective of this thesis is to describe the cellular effects of b-AP15 and explain their occurrence.

More specifically, the aims are:

- Describe the effects of b-AP15 and VLX1570 on cancer cells, specifically in multiple myeloma
- Describe and explain the mechanism of b-AP15-induced mitochondrial damage
- Describe and explain the effects of b-AP15 on aggresome formation and intracellular transport
- Determine whether related natural compounds, sharing a common unsaturated ketone chemical motif, will target the proteasome in a similar fashion to b-AP15
- Confirm USP14 as the cellular target of b-AP15 and describe the mechanism of targeting
- Determine the USP14-dependence of b-AP15 induced proteasome inhibition, and the role of USP14 in proteasomal degradation

Chapter 4

Results

4.1 Paper I: The proteasome deubiquitinase inhibitor VLX1570 shows selectivity for ubiquitin-specific protease-14 and induces apoptosis in multiple myeloma cells

In this paper we have shown that VLX1570 binds to and inhibits the activity of both USP14 and UCHL5, but preferentially binds USP14 both in vitro and in multipe myeloma cell lines. Overall this paper reaffirms the promising anti-proliferative activity of VLX1570/b-AP15 in multiple myeloma, and suggests that the effects seen are mostly due to inhibition of the proteasomal DUB USP14.

We have previously shown that VLX1570, the optimized lead based on the b-AP15 compound, is an inhibitor of the proteasomal DUBs USP14 and UCHL5 [288]. At the time, VLX1570 was in clinical trials for relapsed multiple myeloma in the US. We show that multiple myeloma cell lines treated with VLX1570 show accumulation of poly-ubiquitinated protein substrates at the proteasome, and eventually undergo apoptosis in a time and dose-dependent manner. This included multiple myeloma cells that were resistant to the 20S proteasomal inhibitor bortezomib, and was independent of Bcl-2 status of the cells.

The active site probe Ub-VS showed that USP14 activity was preferentially inhibited by VLX1570, compared to UCHL5, which was supported by similar results using surface plasmon resonance (SPR) and thermostabilisation assays (CETSA). We also show that 20S proteolytic activity is not affected by treatment with VLX1570, nor is poly-ubiquitin association with the proteasomal ubiquitin receptors.

Incubation of USP14 with Ub-VS reduced binding of VLX1570, suggesting that the compound targets the DUB active site. VLX1570 treament induced higher levels of Hsp70B', active caspase 3, Hmox1 and phosphorylated JNK in cells, along with high levels of poly-ubiquitin. Wash-out experiments additionally indicate that VLX1570 is retained in the cell after uptake, and can elicit an apoptotic response several hours later. The pro-apoptotic activity of VLX1570 appears to be independent of the anti-apoptotic effects of Bcl2 , as the compound was still able to induce apoptosis in Bcl2 over-expressing cell lines. It was also unaffected by caspase inhibitors, suggesting a caspase-independent apoptotic pathway.

While VLX1570 was able to induce apoptosis in bortezomib-resistant OPM-2 cells (OPM-2 BZ^R), it displayed a significantly higher IC_{50} value compared to non-resistant cells (See Figure 4.1), with an increase of about 50%, while IC_{50} for bortezomib in (OPM-2 BZ^R) was more than 5-fold that of OPM-2. Liquid scintiallation counter experiments using H^3-VLX1570 suggested that the uptake of the drug was reduced in resistant cells, accounting for the increased tolerance. We also show that OPM-2 BZ^R cells express high levels of anti-apoptotic proteins such as Bcl2. Using a lentivirus transfection system, we were able to show that transfection of different Bcl-2 members could reduce, but not fully eliminated VLX1570-induced apoptosis. Particularly Bcl2-A1 increased VLX1570 tolerance.

VLX1570 was also tested in two xenograft mouse models of multiple myeloma, where it was observed to have anti-neoplastic activity. VLX1570 increased survival in an orthotopic multiple myeloma model using KMS11 cells, while it reduced tumor growth in a subcutaneous model using RPMI8226 cells. The implanted tumors showed increased poly-ubiquitin levels, higher levels of Casapase-3 activation and lower levels of phospho-ERK, suggesting reduced activation of the pro-proliferative Ras/Raf/MEK/ERK pathway [328].

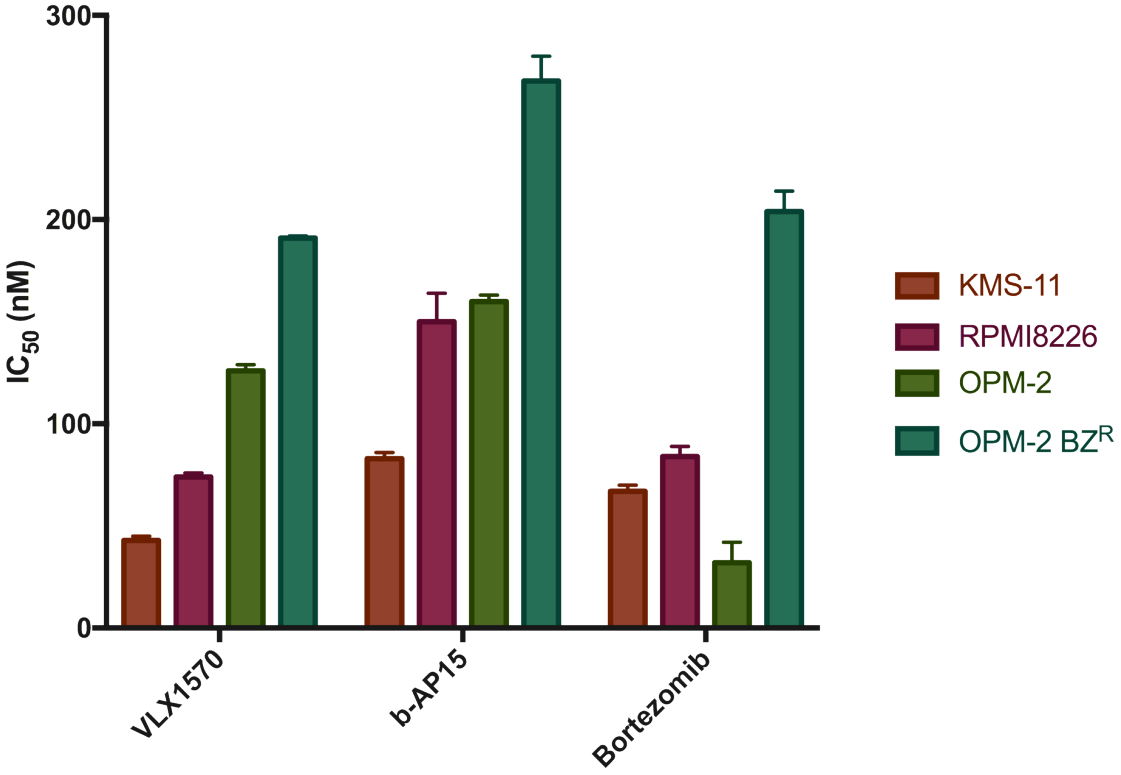


Figure 4.1: IC_{50} of VLX1570, b-AP15 and bortezomib in multiple myeloma cell lines

4.2 Paper II: The deubiquitinase inhibitor b-AP15 induces strong proteotoxic stress and mitochondrial damage

Proteasome inhibition induced by b-AP15 and VLX1570 leads to a build-up of polyubiquitinated and partially misfolded proteins in the cytosol. These misfolded proteins may interact with outer mitochondrial membranes, interfering with normal mitochondrial function, and causing induction of oxidative stress as induced by the mitochondria themselves.

As shown in previous publications, inhibition of the proteasome via blocking of proteasomal DUBs causes cytotoxicity. This cytotoxic effect may be related to oxidative stress, but the mechanism of apoptosis caused by proteasomal DUB inhibition and accumulation of cytosolic protein is not known. Paper II shows that pharmacological inhibition of the proteasome induces proteotoxicity and mitochondrial damage. This effect on mitochondria is associated with the accumulation of misfolded proteins on the outer mitochondrial membrane.

Using RT-PCR and shotgun proteomics we show that the stress response induced by b-AP15 closely resembles that of bortezomib. We observe increases in proteins characteristic of a proteotoxic stress response (Hsp70, JNK, HMOX1 and Hsp40). However, induction of heat shock proteins was elevated in b-AP15-treated cells compared to those treated with bortezomib. These findings are consistent with the higher levels of K48-linked poly-ubiquitin found in cells treated with b-AP15. Isolating poly-ubiquitin chains from treated cells by TUBE assay however, revealed that the average amount of HSP70B' associated with poly-ubiquitin chains in b-AP15 treated cells was decreased compared to bortezomib treatment, suggesting the accumulation of poly-ubiquitinated protein in the cytosol induced by b-AP15 exceeds the cytoprotective capacity of the heat shock response.

Using immunoelectron microscopy and confocal microscopy, we observed that b-AP15 treatment caused a colocalization of K48-ubiquitin and mitochondria. Western blots of isolated mitochondria show a dose-dependent increase of poly-ubiquitin in the mitochondrial fraction of cells treated with b-AP15. Addition of trypsin successfully removed those poly-ubiquitin chains from the mitochondria, suggesting they are associated with the outer mitochondrial membrane only. Co-treatment of cells with CpdA - an inhibitor of the Sec61 translocon that transports unfolded protein into the ER for folding [329] - increased the cytosolic content of poly-ubiquitinated protein, as well as the amount found on mitochondria. Additionally, exposing mitochondria from untreated cells to the cytosol of b-AP15 treated cells also resulted in association of poly-ubiquitin with the outer mitochondrial membrane.

Using a Seahorse XF analyzer, we show that the oxygen consumption rate (OCR), a measure of mitochondrial oxidative phosphorylation, is decreased by exposure to b-AP15. This indicates mitochondrial damage as a result of b-AP15 treatment. However, we observed no depolarization of mitochondrial membranes, nor evidence of mitophagy - the autophagic pathway that removes damaged mitochondria. Autophagic flux in treated cells was not affected by b-AP15 treatment. Consistent with the absence of mitophagy, we observed no relocalization of the E3 ligase Parkin, an essential component of mitophagy [330], to the mitochondria. Instead, we observed the accumulation of the p97/VCP ATPase, a component of the ERAD pathway, possibly in order to

clear the mitochondrial membrane of damaging unfolded proteins.

These results suggest that mitochondrial damage as a result of proteasome inhibition are at least partially responsible for the cytotoxic effects of b-AP15.

4.3 Paper III: Proteasome inhibitor b-AP15 induces enhanced proteotoxicity by inhibiting cytoprotective aggresome formation

In Paper III we show that b-AP15 inhibits ubiquitin dependent aggresome formation in several cancer cell lines. The lack of aggresome formation may explain the enhanced protetoxicity observed with b-AP15 treatment, and suggests the potential use of b-AP15 in cancer treatment as a drug that does not require adjuvant treatment with HDAC inhibitors.

We show here that the proteasomal deubiquitinase inhibitor b-AP15 does not induce cytoprotective aggresome formation, despite inhibition of proteasomal degradation. It has been shown that catalytic inhibition of the proteasome, for example by using bortezomib or MG-132, leads to the build-up of poly-ubiquitin in the cells. The poly-ubiquitinated proteins cluster in a single perinuclear inclusion body called aggresomes. Aggresomes are usually found at the microtubule organizing center (MTOC), and their assembly is mediated by HDAC6, VCP and p62, and dependent on the cytoskeleton. This however, does not occur in cells treated with the non-catalytic proteasome inhibitor b-AP15. Instead, confocal imaging shows that poly-ubiquitin is dispersed in smaller clusters throughout the cytosol. Since aggresome formation is reportedly cytoprotective, we hypothesized that this lack of aggresome formation is the cause of the increased proteotoxicity observed with our line of proteasome deubiquitinase inhibitors, and may explain the mitochondrial toxicity observed in Paper II.

Using the HDAC inhibitor SAHA, we show that inhibition of HDAC6 had no additive effects with b-AP15, while showing strong synergy with bortezomib. Confocal imaging shows that b-AP15 does not cause dissociation of the microtubule network. TUBE assays of isolated poly-ubiquitin show that b-AP15 does not prevent the association of HDAC6, VCP or p62 with poly-ubiquitin. However, treatment with b-AP15 caused a slight increase ubiquitination of HDAC6 itself. Interestingly, our confocal data shows that co-treatment of HeLa cells with b-AP15 and bortezomib also does not result in aggresome formation. The interference of b-AP15 with aggresome formation therefore displays a dominant negative effect over bortezomib, indicating that it acts upstream of substrate trafficking to the aggresome. Consistent with these results our confocal microscopy data shows a rearrangement of clathrin-coated vesicles as well as mitochondria, both of which depend on motor-protein mediated transport for trafficking. Using protetomics data derived from isolated mitochondria, we show that while the mitochondrial transport machinery remains intact, non-mitochondrial proteins are now found associated with mitochondria. This suggests a general transport defect, resulting in a "traffic jam" scenario.

We conclude that b-AP15 has an inhibitory effect on motor-protein dependent transport, which results in cytotoxic protein aggregates remaining in the cytosol, rather than being trafficked and sequestered into the aggresome for autophagic clearance. This explains the enhanced

protetoxic response elicited by b-AP15 treatment, as well as the mitochondrial effects described in Paper II.

4.4 Paper IV: Cytotoxic unsaturated electrophilic compounds commonly target the ubiquitin proteasome system

In this paper, we conducted a drug screen of compounds selected for unsaturated ketone (enone) motifs, such as the α,β- unsaturated ketones found in b-AP15, VLX1570 and G5/2c as well as the natural products curcumin, piperlongumine and gambogic acid [331, 332, 333]. The objective was to determine whether these compounds have a discernible mode of action, despite their proclaimed promiscuity.

Using a panel of 5000 compounds, we performed a drug screen of molecules carrying single or double unsaturated ketone motifs, with the goal of finding and developing other inhibitors of the UPS, primarily of the 19S associated deubiquitinases USP14 and UCHL5. This approach is based on the observed structural elements shared between known 19S DUB inhibitors, such as curcumin, piperlongumine, AC17, b-AP15 and VLX1570, all of which carry one or more Michael acceptors. The screen, using a ubiquitin-YFP (Ub^{G76V}-YFP) expressing reporter cell line, yielded 59 compounds that showed ubiquitin-YFP accumulation, indicating proteasome inhibition. Out of those compounds, 10 induced Ub-YFP in over 50 percent of cells. These 10 compounds were then investigated for their ability to induce K48-Ub accumulation, inhibition of proteolytic activity of the proteasome, and mechanism of induction of apoptosis. A summary of their properties is shown in Table 4.1.

Consistent with proteasome inhibition, the hit compounds induced increased levels of Hsp70B', as well as HMOX1, indicating both proteotoxic and oxidative stress. ER stress marker elF2α and spliced XBP1 were also induced, anlong with apoptotic marker Caspase 3. As with b-AP15, the poly-ubiquitin accumulating in treated cells was shown to cosediment with proteasomes in glycerol gradient fractionation, ruling out inhibition of the Rpn13 proteasomal ubiquitin receptor.

Hit ID	Ub-YFP	USP14 binding	Antineoplastic effects	Developmental toxicity
CB360	++	-	++	-
CB997	++	+	+	+
CB686	++	+		+
CB688	+	+		-
CB113	++	+	++	-
CB729	++	+	+	+
CB742	+	+		+
CB916	++	+	++	-
CB826	++	+	++	+
CB383	++	+		-

Table 4.1: Properties of hit compounds containing α,β-unsaturated ketone motifs

The DUB active site probe Ub-VS was used to test the inhibition of deubiquitinases, revealing inhibition of both 19S DUBs, without effects on other DUBs tested. Association of the several of the compounds with USP14 and UCHL5 was additionally confirmed using Isothermal Calorimetry (ITC), as well as Cellular Extract Thermostabilisation Assay (CETSA). MALDI-TOF mass spectrometry was used to assay molecular mass shifts of USP14 after incubation with hit compounds, and revealed binding for all hits, except CB360. Tryptophan fluorescence showed binding of CB729 and CB916 to the USP14 catalytic domain. Inhibition of cancer cell growth was also confirmed in zebrafish embryos.

Since b-AP15 and VLX1570 are known TrxR inhibitors, all compounds that showed cytotoxic effects were also tested for TrxR inhibition. Some compounds inhibited the proteasome without TrxR inhibition, some inhibited only TrxR and some inhibited only the proteasome. None of the 10 UPS inhibiting hits inhibited TrxR. This confirms that the cytotoxic effects observed with 19S DUB inhibitors are not only due to off-target effects on TrxR and inhibited oxidative stress response, and that inhibition of USP14 and UCHL5 alone is effective in inhibiting cancer growth.

We demonstrate that a substantial fraction (20%) of cytotoxic synthetic compounds containing Michael acceptor groups inhibit proteasome substrate processing and induce a cellular response characteristic of proteasome inhibition. Additionally, biochemical and structural analysis shows inhibition of proteasome-associated cysteine deubiquitinases. We identified a subset of cytotoxic compounds where cell death induction was closely associated with proteasome inhibition. These compounds also showed antineoplastic activity in a zebrafish embryo model, with variable developmental toxicity.

Our findings suggest that proteasome inhibition is a relatively common mode of action by compounds containing Michael acceptor groups. These results explain previous reports on the anti-cancer activity of natural products containing such functional groups, and suggests their use in pharmacological application.

4.5 Paper V: Studies on the specificity of the deubiquitinase inhibitor b-AP15

In this paper we generated a targeted knock-out cell line that is USP14 negative using CrispR/Cas9 generated HCT116 colon carcinoma cells. We used this cell line to determine the dependency of b-AP15 effects on the proteasomal deubiquitinase USP14.

Using the USP14 negative cell line we were able to show that the inhibitory effects of b-AP15 are partially dependent on USP14, further supporting the suggested partial selectivity of compounds containing reactive motifs, such as b-AP15 and the hit compounds described in Paper IV. We do however show that other factors contribute to the proteasome inhibition observed with b-AP15. Our siRNA knockdown of USP14 and UCHL5 show that while removal of a single 19S DUB triggers increases in poly-ubiquitin levels, removal of USP14 alone does not produce as severe an increase as b-AP15 treatment. Dual knockdown of both USP14 and

UCHL5 produced poly-ubiquitin levels more closely resembling those of b-AP15 treatment. This suggests that USP14 inhibition alone cannot account for the effect observed with b-AP15.

USP14$^{-/-}$ cells initially showed a decrease in doubling times, and displayed various morphological changes. Ub-VS labeling of DUBs showed no compensatory increase in other deubiquitinases in USP14$^{-/-}$ cells. Treatment of USP14$^{-/-}$ cells with b-AP15 resulted in a slight increase in K48 poly-ubiquitin levels, while MTT survival assays showed that the USP14$^{-/-}$ cells had a ∼2-fold increase in IC_{50}. USP14 is therefore required to produce the full cellular response to b-AP15.

We designed myc-tagged constructs of USP14 with various mutations of the catalytic triad (C114, H435 and D451), and transiently transfected them into USP14$^{-/-}$ cells. The constructs were well expressed, and the catalytic mutants produced a pronounced increase in poly-ubiquitin levels compared to the knock-out cells. Mutations of the catalytic cysteine (C114A, C114S) resulted in the strongest increase in poly-ubiquitin. Colony formation assays showed that the mutations reduced clonogenic potential, particularly the C114A and C114S mutants. A proposed model explaining the remaining sensitivity of USP14$^{-/-}$ cells to b-AP15 is shown in Figure 4.2.

Without access to a UCHL5$^{-/-}$ cell line, we used a yeast model expressing homologues of the human 19s DUBs. The USP14 homologue ubp6 and the UCHL5 homologue uch2 are both expressed in S. pombe. We used strains defective in one of the deubiquitnases to test for b-AP15 sensitivity. While Ubp6^{+}/Uch2^{-} cells showed increased sensitivity to b-AP15, Ubp6^{-}/Uch2^{+} were less sensitive, consistent with the data from the USP14$^{-/-}$ cell line.

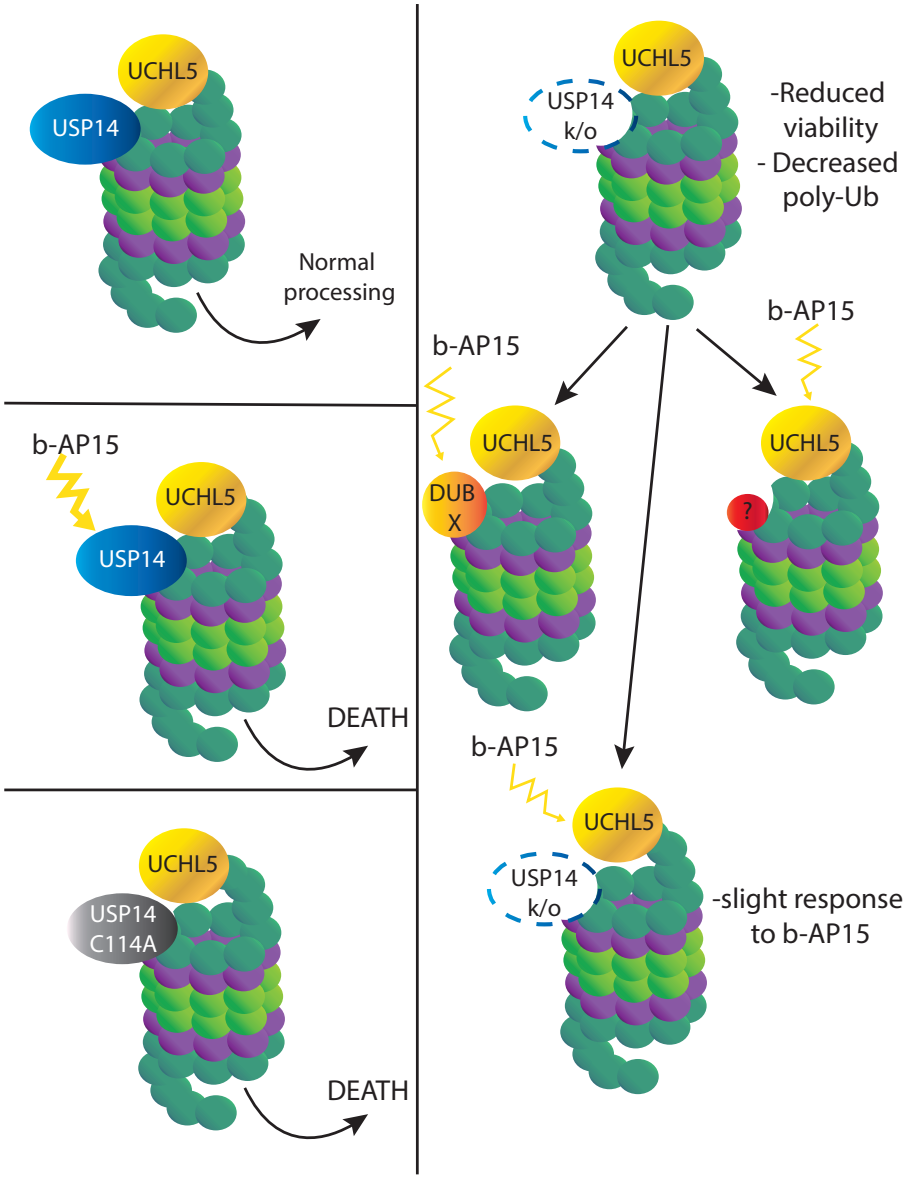


Figure 4.2: Model explaining the remaining partial sensitivity of USP14$^{-/-}$ cells to b-AP15. A likely explanation is that UCHL5 takes over ubiquitin processing following USP14 deletion, and that the remaining sensitivity to b-AP15 can be explained by the previously documented targeting of UCHL5 by b-AP15. Alternatively, a different DUB (DUB X) takes over for USP14, and is likewise affected by b-AP15.

Chapter 5

Discussion

5.1 Cellular effects of b-AP15 and VLX1570

In Paper I we set out to test the binding capabilities and cytotoxic effects of VLX1570, an optimized lead of the proteasomal DUB inhibitor b-AP15. A continuing problem with the b-AP15 compound is its limited solubility, preventing useful clinical application. Derivatives with increased solubility were therefore synthesized and characterized [326]. VLX1570 retains the α-,β-unsaturated ketone structure characteristic of b-AP15, and was therefore expected to similarly target the DUB catalytic cysteines.

Using Ub-VS and SPR binding assays, we show here that VLX1570 displays binding capabilities very similar to those of b-AP15. Like b-AP15 it also appears to be a reversible inhibitor of both USP14 and UCHL5, where it is thought to bind the active site. We consistently show preferential targeting of USP14 over UCHL5. Interestingly, despite SPR data suggesting reversible binding, a washout experiment with VLX1570 indicates that despite removal of the drug from the extracellular medium after only 1h of exposure, the proteasomal DUBs remain inhibited and bound to VLX1570. We observed no recovered DUB activity after 17h of washout. However, we were able to reconcile these results by determining that, like b-AP15 [288], VLX1570 is rapidly taken up into the cells, where it is retained, even upon washout.

Paper I also characterizes the cytotoxic effects displayed by VLX1570, which include induction of Hsp70B' (HSP6A), HMOX1 and JNK, as well as accumulation of K48-linked polyubiquitin. Hsp70B' is a stress-induced chaperone, that is commonly activated by proteasome inhibition [334], HMOX-1 is induced in response to oxidative stress and cellular heat shock conditions [335, 336], and JNK is a component of both intrinsic and extrinsic apoptotic pathways, and is commonly induced by ER stress [210, 337]. Together, this profile of stress activated proteins supports the inhibition of proteasomal degradation by VLX1570, and are consistent with a cellular proteotoxic stress response.

Paper I and several previous publications [322, 323, 324, 325, 338] show that inhibition of the proteasome via blocking of its deubiquitinating activity leads to cellular proteotoxicity. The cytotoxicity of this effect may be related to oxidative stress caused by proteasome inhibition. However, the exact mechanism of the apoptotic effects caused by b-AP15 and its derivative

VLX1570 is not fully understood. Paper II further investigates the mechanism by which b-AP15 triggers apoptosis, and shows that UPS inhibition manifests as proteotoxicity and impaired mitochondrial function.

While we see a reduction in OCR as a result of b-AP15 exposure, as well as severe structural deformation of mitochondria, there is no evidence of mitophagy. Under normal conditions, mitochondrial damage is indicated by a reduction in mitochondrial membrane potential. This depolarization causes the accumulation of the kinase PINK1 on the outer mitochondrial membrane. PINK1 recruits the E3 ligase Parkin to mitochondria. Parkin then marks the mitochondria for autophagic clearance by tagging them with K63-linked ubiquitin chains [330, 339]. This PINK1-Parkin-mitophagy pathway is not activated by b-AP15, despite evidence of mitochondrial damage. Instead we have observed p97/VCP ATPase accumulation on mitochondria. It is possible that p97/VCP is recruited to the mitochondria, in response to b-AP15 treatment, in order to facilitate removal of unfolded and damaging protein, and to restore mitochondrial function.This is supported by our results showing that inhibition of p97/VCP caused higher levels of misfolded proteins to accumulate on mitochondrial membranes, as well as more severe OCR reduction. We have developed a model mechanism to explain this phenomenon (Figure 5.1).

While the details of how b-AP15 causes mitochondrial damage, and the absence of mitophagy despite mitochondrial damage, still remains to be determined, these results suggest that proteasomal inhibition by b-AP15 causes an overload of the heat shock system. This leads to accumulation of partially unfolded proteins in the cytosol. Exposed hydrophobic patches on these proteins may interfere with organelle membranes, including mitochondria.

Cytosolic unfolded and/or poly-ubiquitinated proteins have been shown to sequester into the single large perinuclear inclusion body called the aggresome. Aggresomes are usually found at the MTOC. As a continuation of the work in Paper II, we show in Paper III that no aggresomes form in cells treated with b-AP15. Instead poly-ubiquitin can be seen spread in smaller clusters throughout the cytosol. We hypothesized that since aggresome formation is cytoprotective, the lack of aggresome formation is the cause of the increased proteotoxicity observed with our line of proteasome deubiquitinase inhibitors. Our results have shown that b-AP15 inhibits aggresome formation in a ubiquitin-dependent manner, but that it does not interfere with the recruitment of the aggresome machinery to the poly-ubiquitinated protein. We considered the possibility that the lack of transport of these poly-ubiquitinated proteins was somehow dependent on the chain length of the ubiquitin itself. However, with no way to isolate and examine the contents of b-AP15-induced aggregates, we were unable to pursue this thought further. We show that b-AP15 does not inhibit HDAC6 deacetylation activity, nor does it lead to microtubule hyperacetylation or dissociation. However, treatment with b-AP15 caused a slight increase in poly-ubiquitinated HDAC6 itself. It is possible that poly-ubiquitination of HDAC6 renders the enzyme non-functional in aggresome formation, for example by interfering with the turnover between VCP and HDAC6.

We have also shown that while there are increased levels of poly-ubiquitin chains visible in the cytoplasm following b-AP15 treatment, some of these chains co-precipitate with the

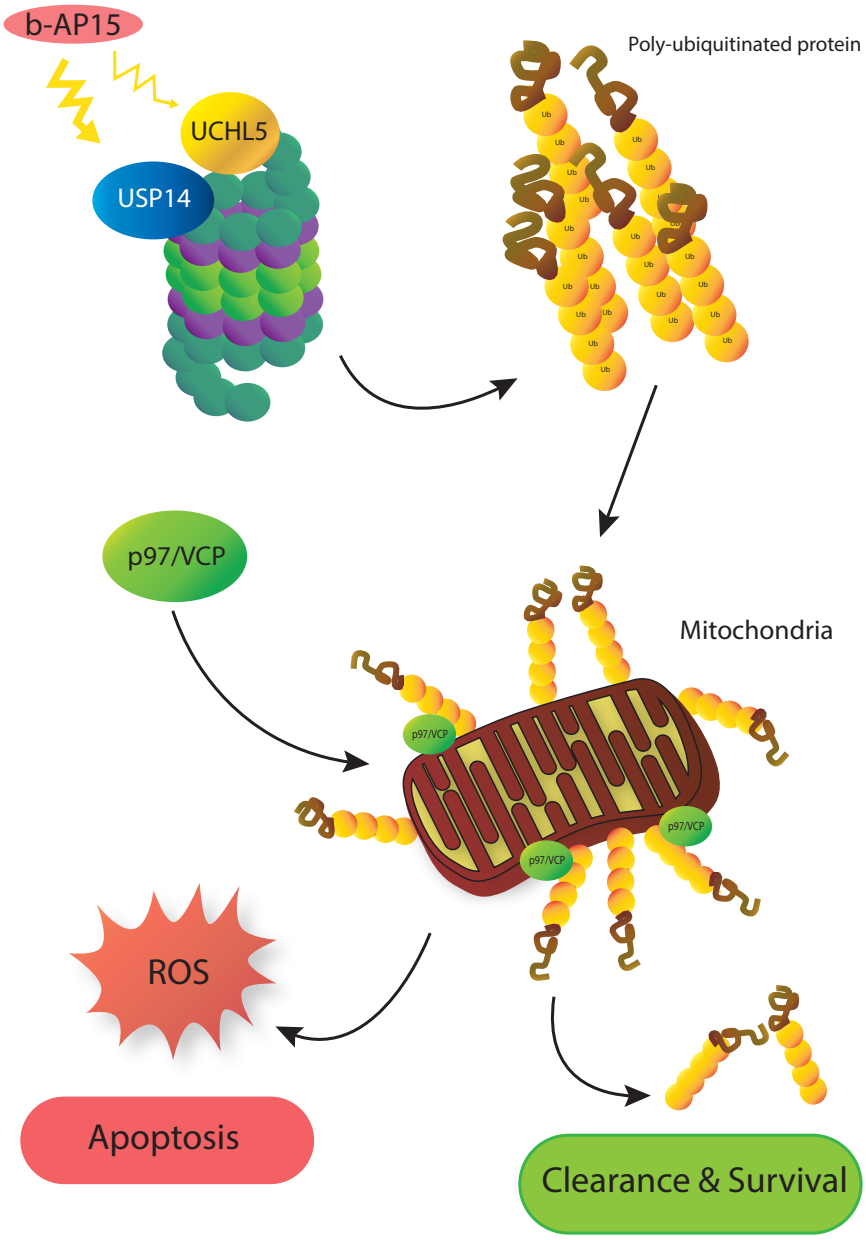


Figure 5.1: Mitochondria-mediated apoptosis in response to b-AP15 treatment. Proteasome inhibition by b-AP15 causes cytosolic accumulation of partially unfolded proteins that interfere with the outer mitochondrial membrane. The ATPase p97/VCP is recruited to clear and restore mitochondria.

proteasome in glycerol gradients, suggesting they are at least transiently associated with the proteasome.

Since we found no obvious defect with components of the aggresome machinery, we investigated whether the trafficking of other substrates of microtubule transport was also affected by b-AP15. We show that already at early timepoints the distribution of clathrin-coated vesicles as well as mitochondria, was affected by b-AP15, indicating a more general defect in trafficking along microtubules.

We suggest that the aggresome defect in b-AP15 treated cells is due to either direct on indirect interference of b-AP15 with motor-protein dependent transport along microtubules. This could either be due to direct action of b-AP15 on a component of transport machinery, or b-AP15 dependent inhibition of a deubiquitinase that is required for trafficking. It appears that both, anterograde and retrograde transport are affected, since protein aggregates are not being transported towards the nucleus, while vesicles and mitochondria appear to cluster in proximity to the nucleus without being moved towards the periphery of the cell. Our tentative model of the effect (shown as Figure 7 in Paper III) proposes a scenario akin to a traffic jam. Without functional transport, trafficking substrates pile up in close proximity of one another, allowing for the damaging interactions of partially unfolded protein with mitochondrial membranes (Paper II), as well as the association of various centrosomal proteins with isolated mitochondria seen in Paper III.

5.2 Biochemical basis of b-AP14 effects and role of USP14

5.2.0 α-,β- unsaturated ketones as DUB inhibitors

A large number of natural compounds have recently been suggested as potential anticancer agents, frequently with unclear or unknown mechanisms of action. Many of these compounds contain functional groups characterized by high chemical reactivity. Researchers and reviewers are often wary of such chemicals, considering them to be too promiscuous to be pharmacologically useful. In fact, identifying which compounds contain so called pan assay interference compounds (PAINS) motifs (i.e. highly reactive functional groups such as unsaturated carbon moieties) is often used to disqualify molecules from drugscreens [340, 341]. Yet, several natural compounds, that have been generating a lot of interest in the phamacological research community, contain PAINS. Examples include piperlongumine [332], gambogic acid [333] and curcumin [331], all of which contain reactive α,β- unsaturated ketones. The α,β-unsaturated ketone motif has repeatedly been reported to be responsible for the phamacological activity of these compounds. This is supported by our findings in Paper I showing that another b-AP15 derivative, VLX1680, with only a single unsaturated carbonyl, displayed lower anti-neoplastic activity than VLX1570 or b-AP15. The α,β-unsaturated ketone motif has even been suggested as a phamacophore model for the development of additional DUB inhibitors [296].This indicates that PAINS do not universally display general cytotoxicity, and can in fact have a certain degree of selectivity, making them pharmacologically useful.

Our DUB inhibitor b-AP15 also contains an α,β-unsaturated ketone motif, and has consistently been shown to selectively target USP14. The presence of the same functional group in a broad spectrum of DUB inhibitors indicates that targeting of DUBs may be a common mechanism for these types of reactive, unsaturated molecules, and motivated the drug screen we performed for Paper IV.

The hits resulting from the screen all contain one or two unsaturated ketones, and display at least partial selectivity for 19S DUBs. Additionally, in docking experiments, the hits consistently mapped into the USP14 active site, and did not visibly inhibit other deubiquitinases. Additionally, of the four hits that did not display developmental toxicity at any dose, three were classified as PAINS, adding another indication that PAINS are not destined to be generally cytotoxic.

Several α-,β-unsaturated ketones, including b-AP15 are known to inhibit thioredoxin reductase 1 (TrxR1), which has an exceptionally reactive catalytic selenocysteine residue, a preferred target of electrophilic compounds. While several of the compounds determined to be UPS inhibitors in Paper IV, also inhibited TrxR, none of the 10 hit compounds did. TrxR inhibition therefore cannot be the main effector of the oxidative and proteotoxic stress observed with these compounds.

Another potential target of these reactive compounds that could result in similar proteotoxic effects is the proteasomal ubiquitin receptor Rpn13. The small molecule inhibitor RA-190, which selectively targets Rpn13 to inhibit proteasomal degradation closely resembles b-AP15 and VLX1570 [342]. This raises the possibility that the observed proteotoxic effects in Paper IV are due to inhibition of ubiquitin binding to the proteasome. We have however repeatedly shown that b-AP15, VLX1570 and all of the 10 hits in Paper IV have no effect on poly-ubiquitin association with the proteasome, ruling out effects on Rpn13.

In addition to the selectivity displayed by b-AP15 [323], VLX1570 [Paper I], and the hit compounds [Paper IV], we show in Paper V that the effects on b-AP15 are primarily dependent on USP14. While there must be some off-target activity to account for the remaining sensitivity to b-AP15 in USP14$^{-/-}$ cells, the primary target through which b-AP15 manifests its proteotoxic effects is USP14.

The unifying conclusion of Paper IPaper IV and Paper V reinforces the potential of b-AP15 and other molecules like it in pharmacological application, particularly in cancer therapy.

5.2.0 USP14 in proteasomal degradation

19S deubiquitinases are confirmed viable targets in cancer therapy. Effects of inhibition consistently show decreased protein degradation, poly-ubiquitin accumulation, proteotoxicity and eventually apoptosis. Their non-genotoxic nature makes small molecule inhibitors of the UCHL5 and USP14 DUBs, such as b-AP15, AC17 and VLX1570, promising candidates for development into cancer treatment.

Targeting USP14 in cancer treatment takes advantage of it pro-proteolytic activity in proteasomal degradation. However, it has been suggested that USP14 actually has the opposite function and actually prevents protein degradation by deubiquitinating substrates and rescu-

ing them from proteolysis. Inhibition of USP14 would then accelerate proteasomal hydrolysis [343]. Several papers have been published supporting either of the two seemingly opposing functions of USP14.

Recent research has suggested that inhibition of USP14 with IU1 increases tau degradation [344], and that upregulation of proteasomal degradation via USP14 inhibition by IU1 impairs autophagic flux [345], suggesting USP14 also works as a regulatory switch between proteolysis and autophagy.

IU1 is the inhibitor most commonly used in the studies investigating enhancing proteolysis via USP14 inhibition [114, 346]. It was recently reported to have been co-crystallized with USP14, where it binds to a unique pocket in proximity to the catalytic triad. Its mechanism of inhibition is therefore steric interference, by preventing access of the ubiquitin C-terminal to the catalytic site of USP14 [347]. This distinguishes it from the α-,β-unsaturated ketone-containing inhibitors, which are thought to interact with the catalytic cysteine, as well as blocking access to the catalytic site (See Figure 5.2B).

Our research offers several counter-indications to the idea that USP14 plays a role in preventing proteasomal degradation. Knockdown of USP14 using siRNA in Paper V showed reduced cellular viablity and increased levels of poly-ubiquitin. Likewise, the USP14$^{-/-}$ cell line displayed slower proliferation, and transient transfection of wt USP14 did not lead to an increase in poly-ubiquitin levels, indicating USP14 does not act to slow down proteasomal degradation. Interestingly, in Paper I we find no evidence of thermostabilization in CETSA assays of USP14 by IU1. This may however be due to dissociation of USP14 from the proteasome at high temperature (discussed in Paper IV). In Paper V we show that treatment with IU1 does in fact lead to an increase in cellular poly-ubiquitin levels, suggesting no enhancement of proteasome activity via the reported inhibition of USP14. We have thus been unable to confirm IU1-dependent enhancement of proteolytic activity, as have other research groups [348]. However, a significant body of evidence indicates that IU1 does in fact specifically target USP14, but does not have cytotoxic effects. This is an indication that b-AP15 must have alternative cellular targets to produce its full effect.

A recent publication took up an intermediate position [349], suggesting that it is the USP14 Ubl domain that promotes proteasomal degradation by binding to the proteasome, independently of USP14 catalytic activity. As part of out USP14 mutant studies in Paper V, we transiently transfected a myc-tagged construct of the USP14-Ubl domain into the HCT116 USP14$^{-/-}$ cell line, but were unable to confirm an increase in proteolytic activity, as poly-ubiquitin levels did not decrease (unpublished data). It is possible that the construct was improperly folded or expressed, and its functionality would need to be confirmed e.g. by native gel electrophoresis before fully trusting these results.

Overall, the data presented in the constituent papers of this thesis supports the role of USP14 as complementary to POH1 activity and an integral component of protein degradation by the proteasome. [111, 113, 350, 351]. The mechanism of ubiquitin-trimming by USP14 at the proteasome, the effects of b-AP15 or IU1, as well as USP14 catalytic mutation or knockout are summarized in Figure 6.2.

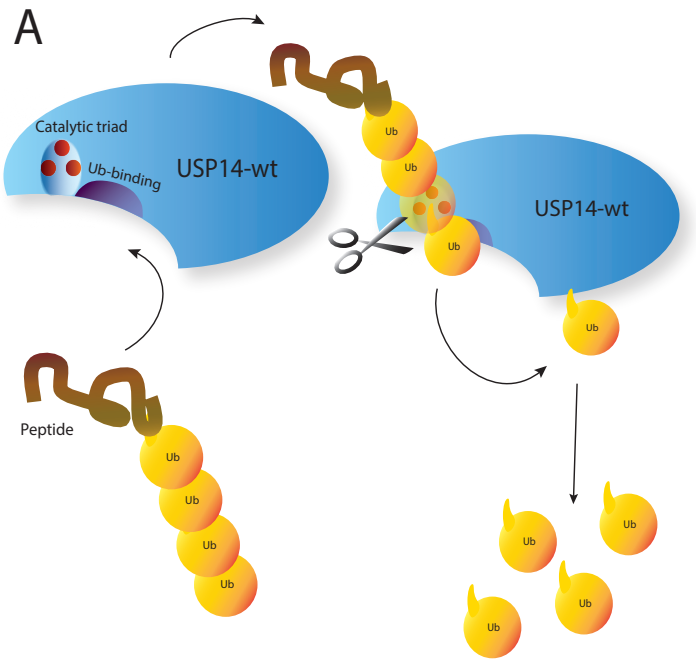
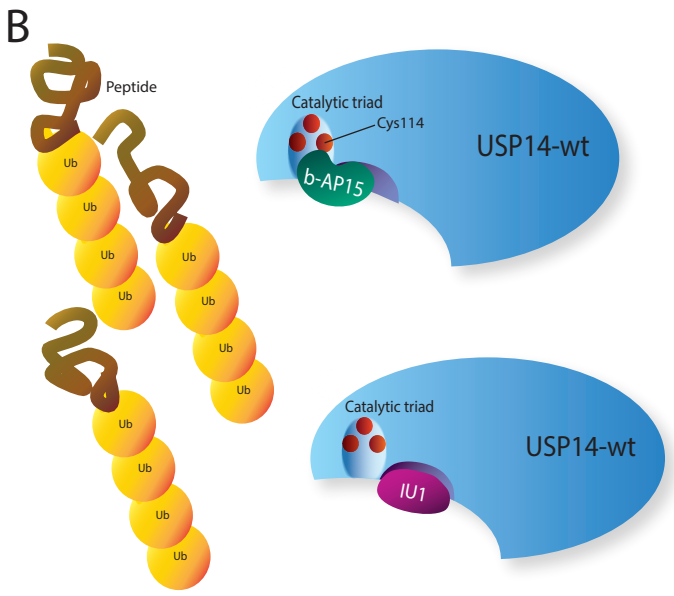
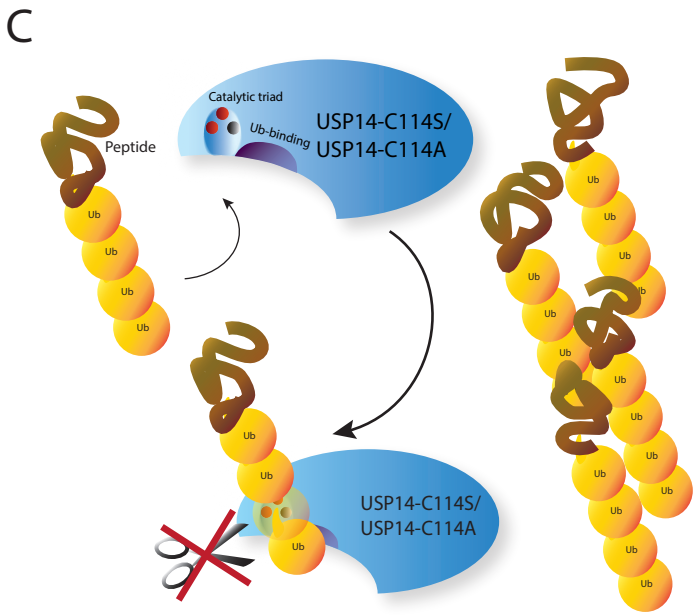


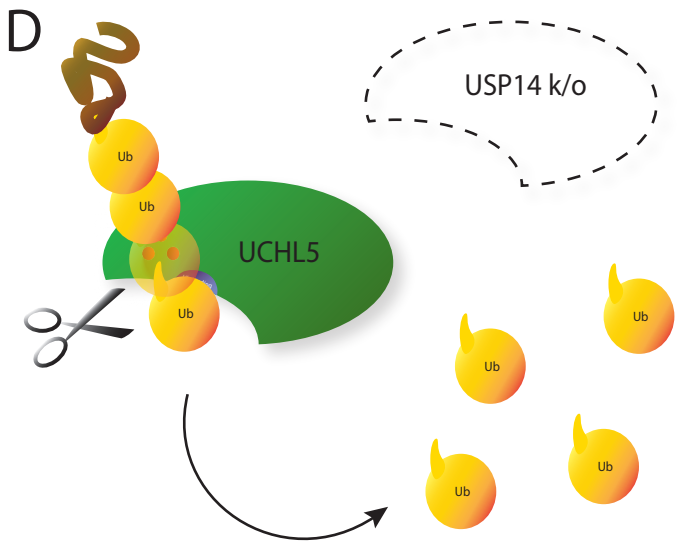
Figure 5.2: **USP14 activity and role in proteasomal degradation** A) Under normal conditions USP14 trims K48-linked poly-ubiquitin chains from the distal end in preparation for substrate degradation by the 26S proteasome



B) Inhibition of USP14 by b-AP15 blocks access of the ubiquitin C-terminal to the catalytic site, while also interfering directly with the catalytic cysteine C114. Inhibition by IU1 sterically blocks ubiquitin access to the catalytic site, without direct contact with the catalytic triad. Ubiquitin chains can no longer bind to USP14 and accumulate in the cytosol.



C) Mutation of the catalytic cysteine C114 to C114A/C114S prevents hydrolysis of the ubiquitin chains, but does not interfere with binding. Poly-ubiquitin chains are not processed, and accumulate in the cytosol.



D) USP14 knockout conditions. Proteasomal deubiquitinating activity is taken over by another DUB (possibly UCHL5), which resolves proteotoxic stress and promotes cell survival

5.3 Ethical Considerations and Limitations

The majority of this project does not involve any patient materials. All work is performed in established cells lines for the cancer types of interest. One concern might be the ethical initial sourcing of those cell lines. In the past there have been conflicts, especially concerning the HeLa cervical cancer cell line. HeLa cells are widely used without credit being given to the original donor Henrietta Lacks, or her family. We have also been using HeLa cells for a selection of experiments, and are aware of these problems. Patient samples - whether it be primary cells or established cell line obtained from patient derived cells - should always be obtained with informed consent, and only under the appropriate ethical permits.
The animal experiments in Paper I were performed by Nerviano Medical Science under the appropriate EU ethical guidelines.

Work carried out exclusively in cell lines has obvious limitations in terms of applicability in humans. It is a very artificial system that in no way resembles the conditions in the human body. Recently attention has also been brought to the issue of reproducibility in medical research, some of which may be due to contamination or mutation of cell lines used in the laboratory. Regularly sequencing cell lines to ensure that they are the correct cells is recommended. Additionally, differences between cell lines also presents issues. Results obtained in one type of cell may not apply in another, making it challenging to determine promising drug candidates to test further.

Chapter 6

Conclusions

The results presented in this thesis contribute to the body of scientific evidence regarding the use of proteasome inhibitors in cancer therapy. Particularly we focus on the applications of inhibitors of the 19S proteasomal ubiquitinases as an alternative to conventional UPS inhibitors such as bortezomib, that have a high incidence of resistance. This body of work adds to our understanding of the cellular effects induced by inhibition of the 19S DUB USP14, as well as the function and interplay of the proteasomal deubiquitinases.

Additionally, we have investigated the potential applications of reactive enone compounds in a phamacological setting. By demonstrating their selectivity, despite being highly reactive, we hope to make a case for continued research into the uses of these types of compounds.

In summary, we conclude the following:

- The small molecule inhibitor b-AP15 targets the 19S deubiquitinase USP14 [Paper I].
- Mitochondrial damage is a direct result of b-AP15 induced protein accumulation in the cytosol [Paper II].
- Proteasome inhibition by b-AP15 does not trigger aggresome formation, leading to enhanced proteotoxicity and mitochodrial damage [Paper III].
- Compounds containing reactive α,β- unsaturated ketones can exhibit partial selectivity for 19S deubiquitinases and have potential pharmacological application [Paper IV].
- USP14 is the primary target of b-AP15, and an effector of b-AP15-induced proteotoxicity [Paper V].

Chapter 7

Future Perspectives

Based on the conclusions presented in this thesis, potential future research directions include:

- Generation and characterization of a UCHL5$^{-/-}$ cell line to confirm results presented in Paper V. Judging by the sensitivity of S.pombe to b-AP15, it can be expected that UCHL5$^{-/-}$ cells will be less sensitive to b-AP15, as they are dependent on USP14 alone.
- Conditional knock-out studies of USP14 in mice. USP14$^{-/-}$ mice have previously been reported to suffer from ataxia [352], but our preliminary results suggest that total homozygous USP14 knockout is lethal *in utero*. We are therefore aiming for a tissue specific knockout of USP14.
- Further investigation of the mechanism by which 19S DUB inhibition induces cell death and interferes with trafficking, particularly the involvement of the motor proteins dynein and kinesin.
- Proteome Mass Spectrometry of the USP14$^{-/-}$ and UCHL5$^{-/-}$ cell lines to determine alterations in ubiquitination patterns, to further elucidate the role of the two DUBs in proteasomal degradation, as well as microarrays to determine altered gene expression.

References

[1] Xin Wang, Magdalena Mazurkiewicz, Ellin-Kristina Hillert, Maria Hägg Olofsson, Stefan Pierrou, Per Hillertz, Joachim Gullbo, Karthik Selvaraju, Aneel Paulus, Sharoon Akhtar, et al. The proteasome deubiquitinase inhibitor VLX1570 shows selectivity for ubiquitin-specific protease-14 and induces apoptosis of multiple myeloma cells. *Scientific Reports*, 6:26979, 2016.

[2] Xiaonan Zhang, Paola Pellegrini, Amir A Saei, Ellin-Kristina Hillert, Magdalena Mazurkiewicz, Maria Hägg Olofsson, Roman A Zubarev, Padraig B D'Arcy, and Stig Linder. The deubiquitinase inhibitor b-AP15 induces strong proteotoxic stress and mitochondrial damage. *Biochemical Pharmacology*, 156:291–301, 2018.

[3] Ellin-Kristina Hillert, Slavica Brjnic, Xiaonan Zhang, Magdalena Mazurkiewicz, Arjan Mofers, Amirata Saei Dibavar, Roman Zubarev, Stig Lider, and Pādraig D'Arcy. Proteasome inhibitor b-AP15 induces enhanced proteotoxicity by inhibiting cytoprotective aggresome formation. *Cancer Letters*, 448:70–83, 2019.

[4] Karthik Selvaraju, Arjan Mofers, Paola Pellegrini, Vivian Morad, Ellin-Kristina Hillert, Belen Espinosa, Elias Arnér, Jonas Malmström, Padraig D'Arcy, Maria Sunnerhagen, and Stig Linder. Targeting of cysteines in proteasome deubiquitinases by diverse sets of compounds containing enones. *Unpublished Manuscript*, 2019.

[5] Ellin-Kristina Hillert, Arjan Mofers, Karthik Selvaraju, Hazal Yilmaz, Paola Pellegrini, Padraig D'Arcy, and Stig Linder. USP14 dependency of cellular response to small molecule inhibitor b-ap15. *Manuscript*, 2019.

[6] Keiji Tanaka. The proteasome: overview of structure and functions. *Proceedings of the Japan Academy, Series B*, 85(1):12–36, 2009.

[7] Ronald Hough, Gregory Pratt, and Martin Rechsteiner. Ubiquitin-lysozyme conjugates. identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. *Journal of Biological Chemistry*, 261(5):2400–2408, 1986.

[8] Robert T Schimke. Control of enzyme levels in mammalian tissues. *Adv Enzymol Relat Areas Mol Biol*, 37(135):1973–187, 1973.

[9] Aaron Ciechanover. The ubiquitin proteolytic system: From a vague idea, through basic mechanisms, and onto human diseases and drug targeting. *Neurology*, 66(1 suppl 1):S7–19, 2006.

[10] Aaron Ciechanover. The unravelling of the ubiquitin system. *Nature Reviews Molecular Cell Biology*, 16(5):322, 2015.

[11] Harold L Segal, Takeo Matsuzawa, Masood Haider, and George J Abraham. What determines the half-life of proteins in, vivo? Some experiences with alanine aminotransferase of rat tissues. *Biochemical and biophysical research communications*, 36(5):764–770, 1969.

[12] Christian De Duve and Robert Wattiaux. Functions of lysosomes. *Annual review of physiology*, 28(1):435–492, 1966.

[13] Masood Haider and Harold L Segal. Some characteristics of the alanine aminotransferase-and arginase-inactivating system of lysosomes. *Archives of biochemistry and biophysics*, 148(1):228–237, 1972.

[14] Nicola T Neff, George N Demartino, and Alfred L Goldberg. The effect of protease inhibitors and decreased temperature on the degradation of different classes of proteins in cultured hepatocytes. *Journal of cellular physiology*, 101(3):439–457, 1979.

[15] Marianne Müller, Wolfgang Dubiel, Jörg Rathmann, and Samuel Rapoport. Determination and characteristics of energy-dependent proteolysis in rabbit reticulocytes. *European journal of biochemistry*, 109(2):405–410, 1980.

[16] Aharon Ciehanover, Yaacov Hod, and Avram Hershko. A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochemical and biophysical research communications*, 81(4):1100–1105, 1978.

[17] Aaron Ciechanover, Hannah Heller, Sarah Elias, Arthur L Haas, and Avram Hershko. ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proceedings of the National Academy of Sciences*, 77(3):1365–1368, 1980.

[18] Avram Hershko, Aaron Ciechanover, Hannah Heller, Arthur L Haas, and Irwin A Rose. Proposed role of ATP in protein breakdown: Conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proceedings of the National Academy of Sciences*, 77(4):1783–1786, 1980.

[19] Avram Hershko, Hannah Heller, Sarah Elias, and Aaron Ciechanover. Components of ubiquitin-protein ligase system: Resolution, affinity purification, and role in protein breakdown. *Journal of Biological Chemistry*, 258(13):8206–8214, 1983.

[20] Avram Hershko, Esther Leshinsky, Dvorah Ganoth, and Hannah Heller. ATP-dependent degradation of ubiquitin-protein conjugates. *Proceedings of the National Academy of Sciences*, 81(6):1619–1623, 1984.

[21] Aaron Ciechanover, Joseph A DiGiuseppe, Beatrice Bercovich, Amir Orian, Joel D Richter, Alan L Schwartz, and Garrett M Brodeur. Degradation of nuclear oncoproteins by the ubiquitin system in vitro. *Proceedings of the National Academy of Sciences*, 88(1):139–143, 1991.

[22] Kenneth L Rock, Colette Gramm, Lisa Rothstein, Karen Clark, Ross Stein, Lawrence Dick, Daniel Hwang, and Alfred L Goldberg. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell*, 78(5):761–771, 1994.

[23] Michael Glotzer, Andrew W Murray, and Marc W Kirschner. Cyclin is degraded by the ubiquitin pathway. *Nature*, 349(6305):132, 1991.

[24] Alexander Varshavsky. Regulated protein degradation. *Trends in biochemical sciences*, 30(6):283–286, 2005.

[25] Aparna Mani and Edward P Gelmann. The ubiquitin-proteasome pathway and its role in cancer. *Journal of Clinical Oncology*, 23(21):4776–4789, 2005.

[26] Deanna M Koepp, J Wade Harper, and Stephen J Elledge. How the cyclin became a cyclin: Regulated proteolysis in the cell cycle. *Cell*, 97(4):431–434, 1999.

[27] Avram Hershko and Aaron Ciechanover. The ubiquitin system. *Annu Rev Biochem*, 67:425–479, 1998.

[28] Alan L Schwartz and Aaron Ciechanover. The ubiquitin-proteasome pathway and pathogenesis of human diseases. *Annual review of medicine*, 50(1):57–74, 1999.

[29] Ido Livneh, Victoria Cohen-Kaplan, Chen Cohen-Rosenzweig, Noa Avni, and Aaron Ciechanover. The life cycle of the 26S proteasome: from birth, through regulation and function, and onto its death. *Cell research*, 26(8):869, 2016.

[30] Lynn Bedford, Simon Paine, Paul W Sheppard, R John Mayer, and Jeroen Roelofs. Assembly, structure, and function of the 26S proteasome. *Trends in cell biology*, 20(7):391–401, 2010.

[31] Julian Adams. The proteasome: Structure, function, and role in the cell. *Cancer treatment reviews*, 29:3–9, 2003.

[32] Engin Özkaynak, Daniel Finley, and Alexander Varshavsky. The yeast ubiquitin gene: head-to-tail repeats encoding a polyubiquitin precursor protein. *Nature*, 312(5995):663, 1984.

[33] Senadhi Vijay-Kumar, Charles E Bugg, and William J Cook. Structure of ubiquitin refined at 1.8 åresolution. *Journal of molecular biology*, 194(3):531–544, 1987.

[34] KD Wilkinson and TK Audhya. Stimulation of ATP-dependent proteolysis requires ubiquitin with the COOH-terminal sequence Arg-Gly-Gly. *Journal of Biological Chemistry*, 256(17):9235–9241, 1981.

[35] A Hershko, H Heller, Esther Eytan, and Y Reiss. The protein substrate binding site of the ubiquitin-protein ligase system. *Journal of Biological Chemistry*, 261(26):11992–11999, 1986.

[36] Andrew G Stephen, Julie S Trausch-Azar, Aaron Ciechanover, and Alan L Schwartz. The ubiquitin-activating enzyme e1 is phosphorylated and localized to the nucleus in a cell cycle-dependent manner. *Journal of Biological Chemistry*, 271(26):15608–15614, 1996.

[37] Christopher E Berndsen and Cynthia Wolberger. New insights into ubiquitin E3 ligase mechanism. *Nature structural & molecular biology*, 21(4):301, 2014.

[38] Raymond J Deshaies and Claudio AP Joazeiro. RING domain E3 ubiquitin ligases. *Annual review of biochemistry*, 78:399–434, 2009.

[39] Rhesa Budhidarmo, Yoshio Nakatani, and Catherine L Day. RINGs hold the key to ubiquitin transfer. *Trends in biochemical sciences*, 37(2):58–65, 2012.

[40] Jon M Huibregtse, Martin Scheffner, Sylvie Beaudenon, and Peter M Howley. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proceedings of the National Academy of Sciences*, 92(7):2563–2567, 1995.

[41] Dawn M Wenzel and Rachel E Klevit. Following ariadne's thread: a new perspective on RBR ubiquitin ligases. *BMC biology*, 10(1):24, 2012.

[42] Cecile M Pickart. Mechanisms underlying ubiquitination. *Annual review of biochemistry*, 70(1):503–533, 2001.

[43] Cecile M Pickart. Back to the future with ubiquitin. *Cell*, 116(2):181–190, 2004.

[44] Linda Hicke. Ubiquitin and proteasomes: Protein regulation by monoubiquitin. *Nature reviews Molecular cell biology*, 2(3):195, 2001.

[45] Linda Hicke and Rebecca Dunn. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annual review of cell and developmental biology*, 19(1):141–172, 2003.

[46] Daniela Hoeller, Nicola Crosetto, Blagoy Blagoev, Camilla Raiborg, Ritva Tikkanen, Sebastian Wagner, Katarzyna Kowanetz, Rainer Breitling, Matthias Mann, Harald Stenmark, et al. Regulation of ubiquitin-binding proteins by monoubiquitination. *Nature cell biology*, 8(2):163, 2006.

[47] David Komander, Francisca Reyes-Turcu, Julien DF Licchesi, Peter Odenwaelder, Keith D Wilkinson, and David Barford. Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. *EMBO reports*, 10(5):466–473, 2009.

[48] Takeshi Tenno, Kenichiro Fujiwara, Hidehito Tochio, Kazuhiro Iwai, E Hayato Morita, Hidenori Hayashi, Shigeo Murata, Hidekazu Hiroaki, Mamoru Sato, Keiji Tanaka, et al. Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin chains. *Genes to Cells*, 9(10):865–875, 2004.

[49] Vincent Chau, John W Tobias, Andreas Bachmair, David Marriott, David J Ecker, David K Gonda, and Alexander Varshavsky. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science*, 243(4898):1576–1583, 1989.

[50] Niki Chondrogianni, Isabelle Petropoulos, Stefanie Grimm, Konstantina Georgila, Betul Catalgol, Bertrand Friguet, Tilman Grune, and Efstathios S Gonos. Protein damage, repair and proteolysis. *Molecular aspects of medicine*, 35:1–71, 2014.

[51] Julia S Thrower, Laura Hoffman, Martin Rechsteiner, and Cecile M Pickart. Recognition of the polyubiquitin proteolytic signal. *The EMBO journal*, 19(1):94–102, 2000.

[52] Guinevere L Grice and James A Nathan. The recognition of ubiquitinated proteins by the proteasome. *Cellular and molecular life sciences*, 73(18):3497–3506, 2016.

[53] Jan-Michael Peters, Werner W Franke, and Jurgen A Kleinschmidt. Distinct 19S and 20S subcomplexes of the 26S proteasome and their distribution in the nucleus and the cytoplasm. *Journal of Biological Chemistry*, 269(10):7709–7718, 1994.

[54] Cezary Wójcik and George N DeMartino. Intracellular localization of proteasomes. *The international journal of biochemistry & cell biology*, 35(5):579–589, 2003.

[55] C Gordon. The intracellular localization of the proteasome. In *The Proteasome—Ubiquitin Protein Degradation Pathway*, pages 175–184. Springer, 2002.

[56] Pia Unverdorben, Florian Beck, Paweł Śledź, Andreas Schweitzer, Günter Pfeifer, Jürgen M Plitzko, Wolfgang Baumeister, and Friedrich Förster. Deep classification of a large cryo-EM dataset defines the conformational landscape of the 26S proteasome. *Proceedings of the National Academy of Sciences*, page 201403409, 2014.

[57] Pia Unverdorben, Florian Beck, Paweł Śledź, Andreas Schweitzer, Günter Pfeifer, Jürgen M Plitzko, Wolfgang Baumeister, and Friedrich Förster. Deep classification of a large cryo-EM dataset defines the conformational landscape of the 26S proteasome. `https://www.rcsb.org/structure/4CR2`, April 2014.

[58] Wolfgang Baumeister, Jochen Walz, Frank Zühl, and Erika Seemüller. The proteasome: paradigm of a self-compartmentalizing protease. *Cell*, 92(3):367–380, 1998.

[59] Michael Groll, Lars Ditzel, Jan Löwe, Daniela Stock, Matthias Bochtler, Hans D Bartunik, and Robert Huber. Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature*, 386(6624):463, 1997.

[60] George N DeMartino and Thomas G Gillette. Proteasomes: machines for all reasons. *Cell*, 129(4):659–662, 2007.

[61] Michael Groll, Monica Bajorek, Alwin Köhler, Luis Moroder, David M Rubin, Robert Huber, Michael H Glickman, and Daniel Finley. A gated channel into the proteasome core particle. *Nature Structural and Molecular Biology*, 7(11):1062, 2000.

[62] James A Brannigan, Guy Dodson, Helen J Duggleby, Peter CE Moody, Janet L Smith, Diana R Tomchick, and Alexey G Murzin. A protein catalytic framework with an n-terminal nucleophile is capable of self-activation. *Nature*, 378(6555):416, 1995.

[63] Masaki Unno, Tsunehiro Mizushima, Yukio Morimoto, Yoshikazu Tomisugi, Keiji Tanaka, Noritake Yasuoka, and Tomitake Tsukihara. The structure of the mammalian 20s proteasome at 2.75 å resolution. *Structure*, 10(5):609–618, 2002.

[64] Michael Groll and Tim Clausen. Molecular shredders: How proteasomes fulfill their role. *Current opinion in structural biology*, 13(6):665–673, 2003.

[65] Matthias Bochtler, Lars Ditzel, Michael Groll, Claudia Hartmann, and Robert Huber. The proteasome. *Annual review of biophysics and biomolecular structure*, 28(1):295–317, 1999.

[66] Christopher Cardozo, Charlene Michaud, and Marian Orlowski. Components of the bovine pituitary multicatalytic proteinase complex (proteasome) cleaving bonds after hydrophobic residues. *Biochemistry*, 38(30):9768–9777, 1999.

[67] Thorsten Wenzel, Christoph Eckerskorn, Friedrich Lottspeich, and Wolfgang Baumeister. Existence of a molecular ruler in proteasomes suggested by analysis of degradation products. *FEBS letters*, 349(2):205–209, 1994.

[68] Tatos N Akopian, Alexei F Kisselev, and Alfred L Goldberg. Processive degradation of proteins and other catalytic properties of the proteasome from Thermoplasma acidophilum. *Journal of Biological Chemistry*, 272(3):1791–1798, 1997.

[69] Alexei F Kisselev, Tatos N Akopian, Kee Min Woo, and Alfred L Goldberg. The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes: Implications for understanding the degradative mechanism and antigen presentation. *Journal of Biological Chemistry*, 274(6):3363–3371, 1999.

[70] Christopher Cardozo, Alexander Vinitsky, Charlene Michaud, and Marian Orlowski. Evidence that the nature of amino acid residues in the p3 position directs substrates to distinct catalytic sites of the pituitary multicatalytic proteinase complex (proteasome). *Biochemistry*, 33(21):6483–6489, 1994.

[71] Michael Groll and Robert Huber. Substrate access and processing by the 20s proteasome core particle. *The international journal of biochemistry & cell biology*, 35(5):606–616, 2003.

[72] Alwin Köhler, Monica Bajorek, Michael Groll, Luis Moroder, David M Rubin, Robert Huber, Michael H Glickman, and Daniel Finley. The substrate translocation channel of the proteasome. *Biochimie*, 83(3-4):325–332, 2001.

[73] George N DeMartino and Clive A Slaughter. The proteasome, a novel protease regulated by multiple mechanisms. *Journal of Biological Chemistry*, 274(32):22123–22126, 1999.

[74] Michael H Glickman, David M Rubin, Olivier Coux, Inge Wefes, Günter Pfeifer, Zdenka Cjeka, Wolfgang Baumeister, Victor A Fried, and Daniel Finley. A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell*, 94(5):615–623, 1998.

[75] Michael H Glickman, David M Rubin, Hongyong Fu, Christopher N Larsen, Olivier Coux, Inge Wefes, Günter Pfeifer, Zdenka Cjeka, Richard Vierstra, Wolfgang Baumeister, et al. Functional analysis of the proteasome regulatory particle. *Molecular biology reports*, 26(1-2):21–28, 1999.

[76] Rina Rosenzweig, Vered Bronner, Daoning Zhang, David Fushman, and Michael H Glickman. Rpn1 and Rpn2 coordinate ubiquitin processing factors at the proteasome. *Journal of Biological Chemistry*, pages jbc–M111, 2012.

[77] Xiang Chen, Byung-Hoon Lee, Daniel Finley, and Kylie J Walters. Structure of proteasome ubiquitin receptor hRpn13 and its activation by the scaffolding protein hRpn2. *Molecular cell*, 38(3):404–415, 2010.

[78] Robert J Tomko, Minoru Funakoshi, Kyle Schneider, Jimin Wang, and Mark Hochstrasser. Heterohexameric ring arrangement of the eukaryotic proteasomal atpases: implications for proteasome structure and assembly. *Molecular cell*, 38(3):393–403, 2010.

[79] Koraljka Husnjak, Suzanne Elsasser, Naixia Zhang, Xiang Chen, Leah Randles, Yuan Shi, Kay Hofmann, Kylie J Walters, Daniel Finley, and Ivan Dikic. Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature*, 453(7194):481, 2008.

[80] Martin Latterich and Sheetal Patel. The aaa team: related atpases with diverse functions. *Trends in cell biology*, 8(2):65–71, 1998.

[81] David M Rubin, Michael H Glickman, Christopher N Larsen, Sadhana Dhruvakumar, and Daniel Finley. Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome. *The EMBO journal*, 17(17):4909–4919, 1998.

[82] George N DeMartino, Carolyn R Moomaw, Olga P Zagnitko, Rita J Proske, Ma Chu-Ping, Steven J Afendis, Jonathan C Swaffield, and Clive A Slaughter. PA700, an ATP-dependent activator of the 20 S proteasome, is an ATPase containing multiple members

of a nucleotide-binding protein family. *Journal of Biological Chemistry*, 269(33):20878–20884, 1994.

[83] Gabriel C Lander, Eric Estrin, Mary E Matyskiela, Charlene Bashore, Eva Nogales, and Andreas Martin. Complete subunit architecture of the proteasome regulatory particle. *Nature*, 482(7384):186, 2012.

[84] Sumit Prakash, Tomonao Inobe, Ace Joseph Hatch, and Andreas Matouschek. Substrate selection by the proteasome during degradation of protein complexes. *Nature chemical biology*, 5(1):29, 2009.

[85] J Wang, Ji-Joon Song, MC Franklin, S Kamtekar, YJ Im, SH Rho, Ihn Sik Seong, CS Lee, Chin Ha Chung, and Soo Hyun Eom. Crystal structures of the HslVU peptidase—ATPase complex reveal an ATP-dependent proteolysis mechanism. *Structure*, 9(2):177–184, 2001.

[86] Cheolju Lee, Michael P Schwartz, Sumit Prakash, Masahiro Iwakura, and Andreas Matouschek. ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. *Molecular cell*, 7(3):627–637, 2001.

[87] Keith D Wilkinson, Valery L Tashayev, Lydia B O'Connor, Christopher N Larsen, Eileen Kasperek, and Cecile M Pickart. Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. *Biochemistry*, 34(44):14535–14546, 1995.

[88] Sucharita Bhattacharyya, Houqing Yu, Carsten Mim, and Andreas Matouschek. Regulated protein turnover: snapshots of the proteasome in action. *Nature reviews Molecular cell biology*, 15(2):122, 2014.

[89] Min Jae Lee, Byung-Hoon Lee, John Hanna, Randall W King, and Daniel Finley. Trimming of ubiquitin chains by proteasome-associated deubiquitinating enzymes. *Molecular & Cellular Proteomics*, 10(5):R110–003871, 2011.

[90] David Komander. Mechanism, specificity and structure of the deubiquitinases. In *Conjugation and Deconjugation of Ubiquitin Family Modifiers*, pages 69–87. Springer, 2010.

[91] David S Leggett, John Hanna, Anna Borodovsky, Bernat Crosas, Marion Schmidt, Rohan T Baker, Thomas Walz, Hidde Ploegh, and Daniel Finley. Multiple associated proteins regulate proteasome structure and function. *Molecular cell*, 10(3):495–507, 2002.

[92] KEiTH D Wilkinson. Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *The FASEB Journal*, 11(14):1245–1256, 1997.

[93] S. M. Nijman, M. P. Luna-Vargas, A. Velds, T. R. Brummelkamp, A. M. Dirac, T. K. Sixma, and R. Bernards. A genomic and functional inventory of deubiquitinating enzymes. *Cell*, 123(5):773–86, 2005.

[94] Charlene Bashore, Corey M Dambacher, Ellen A Goodall, Mary E Matyskiela, Gabriel C Lander, and Andreas Martin. Ubp6 deubiquitinase controls conformational dynamics and substrate degradation of the 26S proteasome. *Nature structural & molecular biology*, 22(9):712, 2015.

[95] K. Lasker, F. Forster, S. Bohn, T. Walzthoeni, E. Villa, P. Unverdorben, F. Beck, R. Aebersold, A. Sali, and W. Baumeister. Molecular architecture of the 26s proteasome holocomplex determined by an integrative approach. *Proc Natl Acad Sci*, 109(5):1380–7, 2012.

[96] Rati Verma, L Aravind, Robert Oania, W Hayes McDonald, John R Yates, Eugene V Koonin, and Raymond J Deshaies. Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science*, 298(5593):611–615, 2002.

[97] Y Amy Lam, Wei Xu, George N DeMartino, and Robert E Cohen. Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. *Nature*, 385(6618):737–740, 1997.

[98] Jun Hamazaki, Shun-ichiro Iemura, Tohru Natsume, Hideki Yashiroda, Keiji Tanaka, and Shigeo Murata. A novel proteasome interacting protein recruits the deubiquitinating enzyme UCH37 to 26S proteasomes. *The EMBO journal*, 25(19):4524–4536, 2006.

[99] Tingting Yao, Ling Song, Jingji Jin, Yong Cai, Hidehisa Takahashi, Selene K Swanson, Michael P Washburn, Laurence Florens, Ronald C Conaway, Robert E Cohen, et al. Distinct modes of regulation of the Uch37 deubiquitinating enzyme in the proteasome and in the Ino80 chromatin-remodeling complex. *Molecular cell*, 31(6):909–917, 2008.

[100] T. K. Maiti, M. Permaul, D. A. Boudreaux, C. Mahanic, S. Mauney, and C. Das. Crystal structure of the catalytic domain of UCHL5, a proteasome-associated human deubiquitinating enzyme, reveals an unproductive form of the enzyme. *FEBS Journal*, 278(24):4917–4926, 2011.

[101] Y Amy Lam, George N DeMartino, Cecile M Pickart, and Robert E Cohen. Specificity of the ubiquitin isopeptidase in the PA700 regulatory complex of 26S proteasomes. *Journal of Biological Chemistry*, 272(45):28438–28446, 1997.

[102] Marie E Morrow, Myung-Il Kim, Judith A Ronau, Michael J Sheedlo, Rhiannon R White, Joseph Chaney, Lake N Paul, Markus A Lill, Katerina Artavanis-Tsakonas, and Chittaranjan Das. Stabilization of an unusual salt bridge in ubiquitin by the extra C-terminal domain of the proteasome-associated deubiquitinase UCH37 as a mechanism of its exo specificity. *Biochemistry*, 52(20):3564–3578, 2013.

[103] Antje Aufderheide, Florian Beck, Florian Stengel, Michaela Hartwig, Andreas Schweitzer, Günter Pfeifer, Alfred L Goldberg, Eri Sakata, Wolfgang Baumeister, and Friedrich Förster. Structural characterization of the interaction of UBP6 with the 26S proteasome. *Proceedings of the National Academy of Sciences*, 112(28):8626–8631, 2015.

[104] Anna Borodovsky, Benedikt M Kessler, Rocco Casagrande, Herman S Overkleeft, Keith D Wilkinson, and Hidde L Ploegh. A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. *The EMBO journal*, 20(18):5187–5196, 2001.

[105] Rina Rosenzweig, Pawel A Osmulski, Maria Gaczynska, and Michael H Glickman. The central unit within the 19S regulatory particle of the proteasome. *Nature structural & molecular biology*, 15(6):573, 2008.

[106] Min Hu, Pingwei Li, Ling Song, Philip D Jeffrey, Tatiana A Chernova, Keith D Wilkinson, Robert E Cohen, and Yigong Shi. Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14. *The EMBO Journal*, 24(21):3747–3756, 2005.

[107] Chueh-Ling Kuo and Alfred Lewis Goldberg. Ubiquitinated proteins promote the association of proteasomes with the deubiquitinating enzyme Usp14 and the ubiquitin ligase Ube3c. *Proceedings of the National Academy of Sciences*, 114(17):E3404–E3413, 2017.

[108] A. Peth, H. C. Besche, and A. L. Goldberg. Ubiquitinated proteins activate the proteasome by binding to USP14/UBP6, which causes 20S gate opening. *Mol Cell*, 36(5):794–804, 2009.

[109] A. Peth, N. Kukushkin, M. Bosse, and A. L. Goldberg. Ubiquitinated proteins activate the proteasomal ATPases by binding to USP14 or UCH37 homologs. *J Biol Chem*, 288(11):7781–90, 2013.

[110] John Hanna, Nathaniel A Hathaway, Yoshiko Tone, Bernat Crosas, Suzanne Elsasser, Donald S Kirkpatrick, David S Leggett, Steven P Gygi, Randall W King, and Daniel Finley. Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasomal degradation. *Cell*, 127(1):99–111, 2006.

[111] Pádraig D'arcy, Slavica Brnjic, Maria Hägg Olofsson, Mårten Fryknäs, Kristina Lindsten, Michelandrea De Cesare, Paola Perego, Behnam Sadeghi, Moustapha Hassan, Rolf Larsson, et al. Inhibition of proteasome deubiquitinating activity as a new cancer therapy. *Nature medicine*, 17(12):1636, 2011.

[112] Pádraig D'Arcy and Stig Linder. Proteasome deubiquitinases as novel targets for cancer therapy. *The international journal of biochemistry & cell biology*, 44(11):1729–1738, 2012.

[113] Padraig D'arcy, Xin Wang, and Stig Linder. Deubiquitinase inhibition as a cancer therapeutic strategy. *Pharmacology & therapeutics*, 147:32–54, 2015.

[114] B. H. Lee, M. J. Lee, S. Park, D. C. Oh, S. Elsasser, P. C. Chen, C. Gartner, N. Dimova, J. Hanna, S. P. Gygi, S. M. Wilson, R. W. King, and D. Finley. Enhancement of proteasome activity by a small-molecule inhibitor of USP14. *Nature*, 467(7312):179–84, 2010.

[115] Jung Hoon Lee, Seung Kyun Shin, Yanxialei Jiang, Won Hoon Choi, Chaesun Hong, Dong-Eun Kim, and Min Jae Lee. Facilitated tau degradation by usp14 aptamers via enhanced proteasome activity. *Scientific reports*, 5:10757, 2015.

[116] Hyoung Tae Kim and Alfred L Goldberg. The deubiquitinating enzyme usp14 allosterically inhibits multiple proteasomal activities and ubiquitin-independent proteolysis. *Journal of Biological Chemistry*, 292:9830–9839, 2017.

[117] Ji Luo, Nicole L Solimini, and Stephen J Elledge. Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell*, 136(5):823–837, 2009.

[118] Raymond J Deshaies. Proteotoxic crisis, the ubiquitin-proteasome system, and cancer therapy. *BMC biology*, 12(1):94, 2014.

[119] Teru Hideshima, Constantine Mitsiades, Masaharu Akiyama, Toshiaki Hayashi, Dharminder Chauhan, Paul Richardson, Robert Schlossman, Klaus Podar, Nikhil C Munshi, Nicholas Mitsiades, et al. Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341. *Blood*, 101(4):1530–1534, 2003.

[120] Patricia Pérez-Galán, Gael Roué, Neus Villamor, Emili Montserrat, Elias Campo, and Dolors Colomer. The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. *Blood*, 107(1):257–264, 2006.

[121] Claudia P Miller, Kechen Ban, Melanie E Dujka, David J McConkey, Mark Munsell, Michael Palladino, and Joya Chandra. NPI-0052, a novel proteasome inhibitor, induces caspase-8 and ROS-dependent apoptosis alone and in combination with HDAC inhibitors in leukemia cells. *Blood*, 110(1):267–277, 2007.

[122] Marian Valko, Dieter Leibfritz, Jan Moncol, Mark TD Cronin, Milan Mazur, and Joshua Telser. Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, 39(1):44–84, 2007.

[123] Kate Samardzic and Kenneth J Rodgers. Oxidised protein metabolism: recent insights. *Biological chemistry*, 398(11):1165–1175, 2017.

[124] Chunpeng Zhu, Wei Hu, Hao Wu, and Xun Hu. No evident dose-response relationship between cellular ROS level and its cytotoxicity–a paradoxical issue in ROS-based cancer therapy. *Scientific reports*, 4:5029, 2014.

[125] Yi-He Ling, Leonard Liebes, Yiyu Zou, and Roman Perez-Soler. Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic response to bortezomib, a novel proteasome inhibitor, in human H460 non-small cell lung cancer cells. *Journal of Biological Chemistry*, 278(36):33714–33723, 2003.

[126] Nihar Ranjan Jana, Priyanka Dikshit, Anand Goswami, and Nobuyuki Nukina. Inhibition of proteasomal function by curcumin induces apoptosis through mitochondrial pathway. *Journal of Biological Chemistry*, 279(12):11680–11685, 2004.

[127] Krishnendu Sinha, Joydeep Das, Pabitra Bikash Pal, and Parames C Sil. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Archives of toxicology*, 87(7):1157–1180, 2013.

[128] Ari Helenius, Thorsten Marquardt, and Ineke Braakman. The endoplasmic reticulum as a protein-folding compartment. *Trends in cell biology*, 2(8):227–231, 1992.

[129] M. Schroder and R. J. Kaufman. Er stress and the unfolded protein response. *Mutat Res*, 569(1-2):29–63, 2005.

[130] Victoria Menendez-Benito, Lisette GGC Verhoef, Maria G Masucci, and Nico P Dantuma. Endoplasmic reticulum stress compromises the ubiquitin–proteasome system. *Human molecular genetics*, 14(19):2787–2799, 2005.

[131] Kevin J Travers, Christopher K Patil, Lisa Wodicka, David J Lockhart, Jonathan S Weissman, and Peter Walter. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell*, 101(3):249–258, 2000.

[132] Andrew Fribley and Cun-Yu Wang. Proteasome inhibitor induces apoptosis through induction of endoplasmic reticulum stress. *Cancer biology & therapy*, 5(7):745–748, 2006.

[133] Wen-Xing Ding, Hong-Min Ni, Wentao Gao, Tamotsu Yoshimori, Donna B Stolz, David Ron, and Xiao-Ming Yin. Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. *The American journal of pathology*, 171(2):513–524, 2007.

[134] Ineke Braakman and Neil J Bulleid. Protein folding and modification in the mammalian endoplasmic reticulum. *Annual review of biochemistry*, 80:71–99, 2011.

[135] M. Schroder and R. J. Kaufman. The mammalian unfolded protein response. *Annu Rev Biochem*, 74:739–89, 2005.

[136] Emanuela Rosati, Rita Sabatini, Filomena De Falco, Beatrice Del Papa, Franca Falzetti, Mauro Di Ianni, Laura Cavalli, Katia Fettucciari, Andrea Bartoli, Isabella Screpanti, et al. γ-secretase inhibitor I induces apoptosis in chronic lymphocytic leukemia cells by proteasome inhibition, endoplasmic reticulum stress increase and notch down-regulation. *International journal of cancer*, 132(8):1940–1953, 2013.

[137] Naoya Mimura, Teru Hideshima, Toshiyasu Shimomura, Rikio Suzuki, Hiroto Ohguchi, Ola Rizq, Shohei Kikuchi, Yasuhiro Yoshida, Francesca Cottini, Jana Jakubikova, et al.

Selective and potent Akt inhibition triggers anti-myeloma activities and enhances fatal endoplasmic reticulum stress induced by proteasome inhibition. *Cancer research*, 74(16):4458–4469, 2014.

[138] Andrey V Cybulsky. The intersecting roles of endoplasmic reticulum stress, ubiquitin–proteasome system, and autophagy in the pathogenesis of proteinuric kidney disease. *Kidney international*, 84(1):25–33, 2013.

[139] Scott A. Houck, Sangita Singh, and Douglas M. Cyr. *Cellular Responses to Misfolded Proteins and Protein Aggregates*, pages 455–461. Humana Press, 2012.

[140] Kezhong Zhang and Randal J Kaufman. Signaling the unfolded protein response from the endoplasmic reticulum. *Journal of Biological Chemistry*, 279(25):25935–25938, 2004.

[141] Anne Bertolotti, Yuhong Zhang, Linda M Hendershot, Heather P Harding, and David Ron. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nature cell biology*, 2(6):326, 2000.

[142] AA McCracken and JL Brodsky. Recognition and delivery of ERAD substrates to the proteasome and alternative paths for cell survival. In *Dislocation and Degradation of Proteins from the Endoplasmic Reticulum*, pages 17–40. Springer, 2006.

[143] Rammohan V Rao, HM Ellerby, and Dale E Bredesen. Coupling endoplasmic reticulum stress to the cell death program. *Cell death and differentiation*, 11(4):372, 2004.

[144] DM Benbrook and A Long. Integration of autography, proteasomal degradation, unfolded protein responce and apoptosis. *Experimental oncology*, 2012.

[145] Grant Miura. ER stress: To live or let die. *Nature chemical biology*, 10(9):695, 2014.

[146] Mary-Jane Gething and Joseph Sambrook. Protein folding in the cell. *Nature*, 355(6355):33, 1992.

[147] Hisae Kadowaki, Hideki Nishitoh, and Hidenori Ichijo. Survival and apoptosis signals in ER stress: the role of protein kinases. *Journal of chemical neuroanatomy*, 28(1-2):93–100, 2004.

[148] Alan G Hinnebusch. The eif-2α kinases: regulators of protein synthesis in starvation and stress. *Seminars in cell biology*, 5(6):417–426, 1994.

[149] Heather P Harding, Yuhong Zhang, and David Ron. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature*, 397(6716):271, 1999.

[150] Heather P Harding, Alisa F Zyryanova, and David Ron. Uncoupling proteostasis and development in vitro with a small molecule inhibitor of the pancreatic endoplasmic reticulum kinase, PERK. *Journal of Biological Chemistry*, 287(53):44338–44344, 2012.

[151] Tom Verfaillie, N Rubio, AD Garg, Geert Bultynck, R Rizzuto, JP Decuypere, Jacques Piette, C Linehan, S Gupta, A Samali, et al. PERK is required at the ER-mitochondrial contact sites to convey apoptosis after ROS-based ER stress. *Cell death and differentiation*, 19(11):1880, 2012.

[152] Neysan Donnelly, Adrienne M Gorman, Sanjeev Gupta, and Afshin Samali. The eIF2α kinases: their structures and functions. *Cellular and molecular life sciences*, 70(19):3493–3511, 2013.

[153] Sara B Cullinan and J Alan Diehl. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *Journal of Biological Chemistry*, 279(19):20108–20117, 2004.

[154] Hiderou Yoshida, Toshie Matsui, Akira Yamamoto, Tetsuya Okada, and Kazutoshi Mori. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell*, 107(7):881–891, 2001.

[155] Kazutoshi Mori. Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell*, 101(5):451–454, 2000.

[156] Birgit Meusser, Christian Hirsch, Ernst Jarosch, and Thomas Sommer. ERAD: the long road to destruction. *Nature cell biology*, 7(8):766, 2005.

[157] Daniel N Hebert, Riccardo Bernasconi, and Maurizio Molinari. ERAD substrates: which way out? *Seminars in cell & developmental biology*, 21(5):526–532, 2010.

[158] Randolph Y Hampton and Thomas Sommer. Finding the will and the way of ERAD substrate retrotranslocation. *Current opinion in Cell Biology*, 24(4):460–466, 2012.

[159] Leticia Lemus and Veit Goder. Regulation of endoplasmic reticulum-associated protein degradation (ERAD) by ubiquitin. *Cells*, 3(3):824–847, 2014.

[160] Hemmo Meyer, Monika Bug, and Sebastian Bremer. Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. *Nature cell biology*, 14(2):117, 2012.

[161] Daniel J Anderson, Ronan Le Moigne, Stevan Djakovic, Brajesh Kumar, Julie Rice, Steve Wong, Jinhai Wang, Bing Yao, Eduardo Valle, Szerenke Kiss von Soly, et al. Targeting the AAA ATPase p97 as an approach to treat cancer through disruption of protein homeostasis. *Cancer cell*, 28(5):653–665, 2015.

[162] Lasse Stach and Paul S Freemont. The AAA+ ATPase p97, a cellular multitool. *Biochemical Journal*, 474(17):2953–2976, 2017.

[163] Ernst Jarosch, Christof Taxis, Corinna Volkwein, Javier Bordallo, Daniel Finley, Dieter H Wolf, and Thomas Sommer. Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nature cell biology*, 4(2):134, 2002.

[164] Shahri Raasi and Dieter H Wolf. Ubiquitin receptors and ERAD: a network of pathways to the proteasome. *Seminars in cell & developmental biology*, 18(6):780–791, 2007.

[165] Ron R Kopito. Aggresomes, inclusion bodies and protein aggregation. *Trends in cell biology*, 10(12):524–530, 2000.

[166] Ronald Wetzel. Mutations and off-pathway aggregation of proteins. *Trends in biotechnology*, 12(5):193–198, 1994.

[167] Anthony L Fink. Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Folding and design*, 3(1):R9–R23, 1998.

[168] Jennifer A Johnston, Cristina L Ward, and Ron R Kopito. Aggresomes: a cellular response to misfolded proteins. *J Cell Biol*, 143(7):1883–1898, 1998.

[169] Christiane Richter-Landsberg. The α-tubulin deacetylase hdac6 in aggresome formation and autophagy: Implications for neurodegeneration. In *Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging*, pages 273–282. Elsevier, 2015.

[170] Yoshiharu Kawaguchi, Jeffrey J Kovacs, Adam McLaurin, Jeffery M Vance, Akihiro Ito, and Tso-Pang Yao. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell*, 115(6):727–738, 2003.

[171] Cyril Boyault, Benoit Gilquin, Yu Zhang, Vladimir Rybin, Elspeth Garman, Wolfram Meyer-Klaucke, Patrick Matthias, Christoph W Müller, and Saadi Khochbin. HDAC6–p97/VCP controlled polyubiquitin chain turnover. *The EMBO journal*, 25(14):3357–3366, 2006.

[172] C. Boyault, K. Sadoul, M. Pabion, and S. Khochbin. HDAC6, at the crossroads between cytoskeleton and cell signaling by acetylation and ubiquitination. *Oncogene*, 26(37):5468–76, 2007.

[173] A. Rodriguez-Gonzalez, T. Lin, A. K. Ikeda, T. Simms-Waldrip, C. Fu, and K. M. Sakamoto. Role of the Aggresome pathway in cancer: Targeting histone deacetylase 6-dependent protein degradation. *Cancer Res*, 68(8):2557–60, 2008.

[174] Nava Zaarur, Anatoli B Meriin, Vladimir L Gabai, and Michael Y Sherman. Triggering aggresome formation:Dissecting aggresome-targting and aggregation signals in Syphilin 1. *Journal of Biological Chemistry*, 283(41):27575–27584, 2008.

[175] Nava Zaarur, Anatoli B Meriin, Eloy Bejarano, Xiaobin Xu, Vladimir L Gabai, Ana Maria Cuervo, and Michael Y Sherman. Proteasome failure promotes positioning of lysosomes around aggresome via local block of microtubule-dependent transport. *Molecular and cellular biology*, 34(7):1336–1348, 2014.

[176] Victoria Cohen-Kaplan, Ido Livneh, Noa Avni, Bertrand Fabre, Tamar Ziv, Yong Tae Kwon, and Aaron Ciechanover. p62- and ubiquitin-dependent stress-induced autophagy of

the mammalian 26S proteasome. *Proceedings of the National Academy of Sciences*, 113(47):E7490–E7499, 2016.

[177] Geir Bjørkøy, Trond Lamark, Andreas Brech, Heidi Outzen, Maria Perander, Aud Øvervatn, Harald Stenmark, and Terje Johansen. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *The Journal of cell biology*, 171(4):603–614, 2005.

[178] Pablo Sánchez-Martín and Masaaki Komatsu. p62/SQSTM1—steering the cell through health and disease. *J Cell Sci*, 131(21):jcs222836, 2018.

[179] Gabriele Zaffagnini, Adriana Savova, Alberto Danieli, Julia Romanov, Shirley Tremel, Michael Ebner, Thomas Peterbauer, Martin Sztacho, Riccardo Trapannone, Abul K Tarafder, et al. p62 filaments capture and present ubiquitinated cargos for autophagy. *The EMBO journal*, 37(5):e98308, 2018.

[180] Ana Maria Cuervo. Breaking down autophagy. *Trends Cell Biol*, 6(9):637–638, 2016.

[181] Beth Levine and Daniel J Klionsky. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Developmental cell*, 6(4):463–477, 2004.

[182] Zhangyuan Yin, Clarence Pascual, and Daniel J Klionsky. Autophagy: machinery and regulation. *Microbial Cell*, 3(12):588, 2016.

[183] Noboru Mizushima, Beth Levine, Ana Maria Cuervo, and Daniel J Klionsky. Autophagy fights disease through cellular self-digestion. *Nature*, 451(7182):1069, 2008.

[184] Viktor I Korolchuk, Fiona M Menzies, and David C Rubinsztein. Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. *FEBS letters*, 584(7):1393–1398, 2010.

[185] Trond Lamark and Terje Johansen. Autophagy: links with the proteasome. *Current Opinion in Cell Biology*, 22(2):192–198, 2010.

[186] Huabo Su and Xuejun Wang. p62 stages an interplay between the ubiquitin-proteasome system and autophagy in the heart of defense against proteotoxic stress. *Trends in cardiovascular medicine*, 21(8):224–228, 2011.

[187] A. Iwata, B. E. Riley, J. A. Johnston, and R. R. Kopito. Hdac6 and microtubules are required for autophagic degradation of aggregated huntingtin. *J Biol Chem*, 280(48):40282–92, 2005.

[188] Mohamed-Amine Hamouda, Nathalie Belhacene, Alexandre Puissant, Pascal Colosetti, Guillaume Robert, Arnaud Jacquel, Bernard Mari, Patrick Auberger, and Frederic Luciano. The small heat shock protein B8 (HSPB8) confers resistance to bortezomib by promoting autophagic removal of misfolded proteins in multiple myeloma cells. *Oncotarget*, 5(15):6252, 2014.

[189] Wen-Xing Ding and Xiao-Ming Yin. Sorting, recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome. *Autophagy*, 4(2):141–150, 2008.

[190] L Ouyang, Z Shi, S Zhao, F-T Wang, T-T Zhou, B Liu, and J-K Bao. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell proliferation*, 45(6):487–498, 2012.

[191] Keyi Zhu, Kenneth Dunner Jr, and David J McConkey. Proteasome inhibitors activate autophagy as a cytoprotective response in human prostate cancer cells. *Oncogene*, 29(3):451, 2010.

[192] Junghyun Lim, M Lenard Lachenmayer, Shuai Wu, Wenchao Liu, Mondira Kundu, Rong Wang, Masaaki Komatsu, Young J Oh, Yanxiang Zhao, and Zhenyu Yue. Proteotoxic stress induces phosphorylation of p62/SQSTM1 by ULK1 to regulate selective autophagic clearance of protein aggregates. *PLoS genetics*, 11(2):e1004987, 2015.

[193] Gabriele Zaffagnini, Adriana Savova, Alberto Danieli, Julia Romanov, Shirley Tremel, Michael Ebner, Thomas Peterbauer, Martin Sztacho, Riccardo Trapannone, Abul K Tarafder, et al. Phasing out the bad—how SQSTM1/p62 sequesters ubiquitinated proteins for degradation by autophagy. *Autophagy*, 14(7):1280–1282, 2018.

[194] Daxiao Sun, Rongbo Wu, Jingxiang Zheng, Pilong Li, and Li Yu. Polyubiquitin chain-induced p62 phase separation drives autophagic cargo segregation. *Cell research*, page 1, 2018.

[195] Ilaria Bellezza, Ileana Giambanco, Alba Minelli, and Rosario Donato. Nrf2-Keap1 signaling in oxidative and reductive stress. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1865(5):721–733, 2018.

[196] David G Breckenridge, Marc Germain, Jaigi P Mathai, Mai Nguyen, and Gordon C Shore. Regulation of apoptosis by endoplasmic reticulum pathways. *Oncogene*, 22(53):8608, 2003.

[197] Wen-Xing Ding, Hong-Min Ni, Wentao Gao, Xiaoyun Chen, Jeong Han Kang, Donna B Stolz, Jinsong Liu, and Xiao-Ming Yin. Oncogenic transformation confers a selective susceptibility to the combined suppression of the proteasome and autophagy. *Molecular cancer therapeutics*, 8(7):2036–2045, 2009.

[198] Yi-He Ling, Leonard Liebes, Jian-Dong Jiang, James F Holland, Peter J Elliott, Julian Adams, Franco M Muggia, and Roman Perez-Soler. Mechanisms of proteasome inhibitor PS-341-induced G2-M-phase arrest and apoptosis in human non-small cell lung cancer cell lines. *Clinical Cancer Research*, 9(3):1145–1154, 2003.

[199] Miyashita Toshiyuki and John C Reed. Tumor suppressor p53 is a direct transcriptional activator of the human Bax gene. *Cell*, 80(2):293–299, 1995.

[200] Hirohito Yamaguchi and Hong-Gang Wang. CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. *Journal of Biological Chemistry*, 279(44):45495–45502, 2004.

[201] Tomohiro Yorimitsu, Usha Nair, Zhifen Yang, and Daniel J Klionsky. Endoplasmic reticulum stress triggers autophagy. *Journal of Biological Chemistry*, 281(40):30299–30304, 2006.

[202] Raffaella Iurlaro, Franziska Püschel, Clara Lucía León-Annicchiarico, Hazel O'Connor, Seamus J Martin, Daniel Palou-Gramón, Estefanía Lucendo, and Cristina Muñoz Pinedo. Glucose deprivation induces ATF4-mediated apoptosis through TRAIL death receptors. *Molecular and cellular biology*, 37(10):e00479–16, 2017.

[203] Albert F Kabore, Jinmie Sun, Xiaojie Hu, Kristin McCrea, James B Johnston, and Spencer B Gibson. The trail apoptotic pathway mediates proteasome inhibitor induced apoptosis in primary chronic lymphocytic leukemia cells. *Apoptosis*, 11(7):1175–1193, 2006.

[204] Ricky W Johnstone, Ailsa J Frew, and Mark J Smyth. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nature Reviews Cancer*, 8(10):782, 2008.

[205] S1 Oyadomari and M Mori. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell death and differentiation*, 11(4):381, 2004.

[206] Matthew H Brush, Douglas C Weiser, and Shirish Shenolikar. Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1α to the endoplasmic reticulum and promotes dephosphorylation of the α subunit of eukaryotic translation initiation factor 2. *Molecular and cellular biology*, 23(4):1292–1303, 2003.

[207] X Chen and X-M Yin. Coordination of autophagy and the proteasome in resolving endoplasmic reticulum stress. *Veterinary pathology*, 48(1):245–253, 2011.

[208] Yongjie Wei, Sangita C Sinha, and Beth Levine. Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation. *Autophagy*, 4(7):949–951, 2008.

[209] Nicole C McKnight and Zhenyu Yue. Beclin 1, an essential component and master regulator of PI3K-III in health and disease. *Current pathobiology reports*, 1(4):231–238, 2013.

[210] Danny N Dhanasekaran and E Premkumar Reddy. JNK signaling in apoptosis. *Oncogene*, 27(48):6245, 2008.

[211] Meng Lu, Chiara Boschetti, and Alan Tunnacliffe. Long term aggresome accumulation leads to DNA damage, p53-dependent cell cycle arrest, and steric interference in mitosis. *Journal of Biological Chemistry*, 290(46):27986–28000, 2015.

[212] Laurence Catley, Ellen Weisberg, Tanyel Kiziltepe, Yu-Tzu Tai, Teru Hideshima, Paola Neri, Pierfrancesco Tassone, Peter Atadja, Dharminder Chauhan, Nikhil C Munshi, et al. Aggresome induction by proteasome inhibitor bortezomib and α-tubulin hyperacetylation by tubulin deacetylase (TDAC) inhibitor LBH589 are synergistic in myeloma cells. *Blood*, 108(10):3441–3449, 2006.

[213] Seiichiro Komatsu, Shota Moriya, Xiao-Fang Che, Tomohisa Yokoyama, Norio Kohno, and Keisuke Miyazawa. Combined treatment with SAHA, bortezomib, and clarithromycin for concomitant targeting of aggresome formation and intracellular proteolytic pathways enhances ER stress-mediated cell death in breast cancer cells. *Biochemical and biophysical research communications*, 437(1):41–47, 2013.

[214] Mikiei Tanaka, Yong Man Kim, Gwang Lee, Eunsung Junn, Takeshi Iwatsubo, and M Maral Mouradian. Aggresomes formed by α-synuclein and synphilin-1 are cytoprotective. *Journal of Biological Chemistry*, 279(6):4625–4631, 2004.

[215] S. T. Nawrocki, J. S. Carew, M. S. Pino, R. A. Highshaw, R. H. Andtbacka, Jr. Dunner, K., A. Pal, W. G. Bornmann, P. J. Chiao, P. Huang, H. Xiong, J. L. Abbruzzese, and D. J. McConkey. Aggresome disruption: a novel strategy to enhance bortezomib-induced apoptosis in pancreatic cancer cells. *Cancer Res*, 66(7):3773–81, 2006.

[216] Teru Hideshima, James E Bradner, Jason Wong, Dharminder Chauhan, Paul Richardson, Stuart L Schreiber, and Kenneth C Anderson. Small-molecule inhibition of proteasome and aggresome function induces synergistic antitumor activity in multiple myeloma. *Proceedings of the National Academy of Sciences of the United States of America*, 102(24):8567–8572, 2005.

[217] David J McConkey, Matthew White, and Wudan Yan. Hdac inhibitor modulation of proteotoxicity as a therapeutic approach in cancer. In *Advances in cancer research*, volume 116, pages 131–163. Elsevier, 2012.

[218] Dan T Vogl, Parameswaran N Hari, Sundar Jagannath, Simon S Jones, Jeffrey G Supko, Gina Leone, Catherine Wheeler, Robert Z Orlowski, Paul G Richardson, and Sagar Lonial. ACY-1215, a selective histone deacetylase (HDAC) 6 inhibitor: interim results of combination therapy with bortezomib in patients with multiple myeloma (MM), 2013.

[219] Paul G Richardson, Constantine S Mitsiades, Jacob P Laubach, Roman Hajek, Ivan Spicka, Meletios A Dimopoulos, Philippe Moreau, David S Siegel, Sundar Jagannath, and Kenneth C Anderson. Preclinical data and early clinical experience supporting the use of histone deacetylase inhibitors in multiple myeloma. *Leukemia research*, 37(7):829–837, 2013.

[220] Shota Moriya, Seiichiro Komatsu, Kaho Yamasaki, Yusuke Kawai, Hiroko Kokuba, Ayako Hirota, Xiao-Fang Che, Masato Inazu, Akihiko Gotoh, Masaki Hiramoto, et al. Targeting the integrated networks of aggresome formation, proteasome, and autophagy potentiates

ER stress-mediated cell death in multiple myeloma cells. *International journal of oncology*, 46(2):474–486, 2015.

[221] S Kawabata, JJ Gills, JR Mercado-Matos, J Lopiccolo, W Wilson III, MC Hollander, and PA Dennis. Synergistic effects of nelfinavir and bortezomib on proteotoxic death of NSCLC and multiple myeloma cells. *Cell death & disease*, 3(7):e353, 2012.

[222] Steven Grant. Enhancing proteotoxic stress as an anticancer strategy. *Oncotarget*, 2(4):284, 2011.

[223] Nickolay Neznanov, Andrei P Komarov, Lubov Neznanova, Patricia Stanhope-Baker, and Andrei V Gudkov. Proteotoxic stress targeted therapy (PSTT): induction of protein misfolding enhances the antitumor effect of the proteasome inhibitor bortezomib. *Oncotarget*, 2(3):209, 2011.

[224] Hwan-Ching Tai and Erin M Schuman. Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. *Nature Reviews Neuroscience*, 9(11):826, 2008.

[225] L Eric Huang, Jie Gu, Maureen Schau, and H Franklin Bunn. Regulation of hypoxia-inducible factor 1α is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proceedings of the National Academy of Sciences*, 95(14):7987–7992, 1998.

[226] Lakshman Gunaratnam, Melissa Morley, Aleksandra Franovic, Natalie De Paulsen, Karim Mekhail, Doris AE Parolin, Eijiro Nakamura, Ian AJ Lorimer, and Stephen Lee. Hypoxia inducible factor activates the transforming growth factor-α/epidermal growth factor receptor growth stimulatory pathway in VHL-/-renal cell carcinoma cells. *Journal of Biological Chemistry*, 278(45):44966–44974, 2003.

[227] Nancie Petrucelli, Mary B Daly, and Tuya Pal. BRCA1-and BRCA2-associated hereditary breast and ovarian cancer. *GeneReviews*, 2016.

[228] Peter S Brzovic, Jose E Meza, Mary-Claire King, and Rachel E Klevit. BRCA1-RING domain cancer-predisposing mutations: Structural consequences and effects on protein-protein interactions. *Journal of Biological Chemistry*, 2001.

[229] Arnold J Levine. p53, the cellular gatekeeper for growth and division. *Cell*, 88(3):323–331, 1997.

[230] Sandra L Harris and Arnold J Levine. The p53 pathway: positive and negative feedback loops. *Oncogene*, 24(17):2899, 2005.

[231] Karen H Vousden and David P Lane. p53 in health and disease. *Nature reviews Molecular cell biology*, 8(4):275, 2007.

[232] Reiko Honda, Hirofumi Tanaka, and Hideyo Yasuda. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS letters*, 420(1):25–27, 1997.

[233] Jamil Momand, David Jung, Sharon Wilczynski, and Joyce Niland. The MDM2 gene amplification database. *Nucleic acids research*, 26(15):3453–3459, 1998.

[234] Tim Crook, Karen H Vousden, and John A Tidy. Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. *Cell*, 67(3):547–556, 1991.

[235] Xianqiang Li and Philip Coffino. High-risk human papillomavirus E6 protein has two distinct binding sites within p53, of which only one determines degradation. *Journal of Virology*, 70(7):4509–4516, 1996.

[236] X Liu, H Yuan, B Fu, GL Disbrow, T Apolinario, V Tomaic, ML Kelley, CC Baker, J Huibregtse, and R Schlegel. The E6-AP ubiquitin ligase is required for transactivation of the hTERT promoter by the human papillomavirus E6 oncoprotein. *The Journal of biological chemistry*, 280(11):10807, 2005.

[237] Michele Pagano, Sun W Tam, Anne M Theodoras, Peggy Beer-Romero, Giannino Del Sal, Vincent Chau, P Renée Yew, Giulio F Draetta, and Mark Rolfe. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science*, 269(5224):682–685, 1995.

[238] Michiko Shirane, Yumiko Harumiya, Noriko Ishida, Aizan Hirai, Chikara Miyamoto, Shigetsugu Hatakeyama, Kei-ichi Nakayama, and Masatoshi Kitagawa. Down-regulation of p27/Kip1 by two mechanisms, ubiquitin-mediated degradation and proteolytic processing. *Journal of Biological Chemistry*, 274(20):13886–13893, 1999.

[239] Kiichiro Tomoda, Yukiko Kubota, and Jun-ya Kato. Degradation of the cyclin-dependent-kinase inhibitor p27-Kip1 is instigated by Jab1. *Nature*, 398(6723):160, 1999.

[240] Roberto Chiarle, Leo M Budel, Jeffrey Skolnik, Glauco Frizzera, Marco Chilosi, Alessandra Corato, Gianni Pizzolo, Jory Magidson, Alessia Montagnoli, Michele Pagano, et al. Increased proteasome degradation of cyclin-dependent kinase inhibitor p27 is associated with a decreased overall survival in mantle cell lymphoma. *Blood*, 95(2):619–626, 2000.

[241] Massimo Loda, Barry Cukor, Sun W Tam, Philip Lavin, Michelangelo Fiorentinc, Glulio F Draetta, J Milburn Jessup, and Michele Pagano. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nature medicine*, 3(2):231, 1997.

[242] Jeannette Philipp-Staheli, Shannon R Payne, and Christopher J Kemp. p27-Kip1: regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. *Experimental cell research*, 264(1):148–168, 2001.

[243] Valery Sudakin, DVORAH Ganoth, Aviva Dahan, Hannah Heller, Judith Hershko, Francis C Luca, Joan V Ruderman, and Avram Hershko. The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. *Molecular biology of the cell*, 6(2):185–197, 1995.

[244] Khandan Keyomarsi, Jr D Conte, Wendy Toyofuku, and M Pat Fox. Deregulation of cyclin e in breast cancer. *Oncogene*, 11(5):941–950, 1995.

[245] Khandan Keyomarsi, Susan L Tucker, Thomas A Buchholz, Matthew Callister, YE Ding, Gabriel N Hortobagyi, Isabelle Bedrosian, Christopher Knickerbocker, Wendy Toyofuku, Michael Lowe, et al. Cyclin E and survival in patients with breast cancer. *New England Journal of Medicine*, 347(20):1566–1575, 2002.

[246] Martin Erlanson and Göran Landberg. Prognostic implications of p27 and cyclin E protein contents in malignant lymphomas. *Leukemia & lymphoma*, 40(5-6):461–470, 2001.

[247] Rongbin Wei, Xiaodong Liu, Weixin Yu, Tianshu Yang, Wenping Cai, Junjun Liu, Xiao Huang, Guo-tong Xu, Shouliang Zhao, Jianhua Yang, et al. Deubiquitinases in cancer. *Oncotarget*, 6(15):12872, 2015.

[248] Roland Pfoh, Ira Kay Lacdao, and Vivian Saridakis. Deubiquitinases and the new therapeutic opportunities offered to cancer. *Endocrine-related cancer*, 22(1):T35–T54, 2015.

[249] Seiichi Shinji, Zenya Naito, Shunji Ishiwata, Toshiyuki Ishiwata, Noritake Tanaka, Kiyonori Furukawa, Hideyuki Suzuki, Tomoko Seya, Akihisa Matsuda, Miwako Katsuta, et al. Ubiquitin-specific protease 14 expression in colorectal cancer is associated with liver and lymph node metastases. *Oncology reports*, 15(3):539–543, 2006.

[250] Robert Z Orlowski and Deborah J Kuhn. Proteasome inhibitors in cancer therapy: lessons from the first decade. *Clinical cancer research*, 14(6):1649–1657, 2008.

[251] AL Goldberg. Functions of the proteasome: From protein degradation and immune surveillance to cancer therapy. *Biochemical Society Transactions*, 35(1):12–17, 2007.

[252] Fangbao Ding, Haibo Xiao, Mingsong Wang, Xiao Xie, and Fengqing Hu. The role of the ubiquitin-proteasome pathway in cancer development and treatment. *Front biosci (landmark Ed)*, 19:886–895, 2014.

[253] Di Chen and Q Ping Dou. The ubiquitin-proteasome system as a prospective molecular target for cancer treatment and prevention. *Current Protein and Peptide Science*, 11(6):459–470, 2010.

[254] Julian Adams, Vito J Palombella, Edward A Sausville, Jill Johnson, Antonia Destree, Douglas D Lazarus, Jochen Maas, Christine S Pien, Samuel Prakash, and Peter J Elliott. Proteasome inhibitors: A novel class of potent and effective antitumor agents. *Cancer research*, 59(11):2615–2622, 1999.

[255] Elisabet E Manasanch and Robert Z Orlowski. Proteasome inhibitors in cancer therapy. *Nature reviews Clinical oncology*, 14(7):417, 2017.

[256] Kush Patel, Zainab SO Ahmed, Xuemei Huang, Qianqian Yang, Elmira Ekinci, Christine M Neslund-Dudas, Bharati Mitra, Fawzy AEM Elnady, Young-Hoon Ahn, Huanjie Yang, et al. Discovering proteasomal deubiquitinating enzyme inhibitors for cancer therapy: Lessons from rational design, nature and old drug reposition. *Future medicinal chemistry*, 10(17):2087–2108, 2018.

[257] Wei Zhang and Sachdev S Sidhu. Development of inhibitors in the ubiquitination cascade. *FEBS letters*, 588(2):356–367, 2014.

[258] Michael R Mattern, Jian Wu, and Benjamin Nicholson. Ubiquitin-based anticancer therapy: carpet bombing with proteasome inhibitors vs surgical strikes with E1, E2, E3, or DUB inhibitors. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1823(11):2014–2021, 2012.

[259] Guy S Salvesen and Colin S Duckett. IAP proteins: blocking the road to death's door. *Nature reviews Molecular cell biology*, 3(6):401, 2002.

[260] Kristina Bielskienė, Lida Bagdonienė, Julija Mozūraitienė, Birutė Kazbarienė, and Ernestas Janulionis. E3 ubiquitin ligases as drug targets and prognostic biomarkers in melanoma. *Medicina*, 51(1):1–9, 2015.

[261] Yi Sun. Targeting E3 ubiquitin ligases for cancer therapy. *Cancer biology & therapy*, 2(6):623–629, 2003.

[262] Rati Verma, Noel R Peters, Mariapina D'onofrio, Gregory P Tochtrop, Kathleen M Sakamoto, Ranjani Varadan, Mingsheng Zhang, Philip Coffino, David Fushman, Raymond J Deshaies, et al. Ubistatins inhibit proteasome-dependent degradation by binding the ubiquitin chain. *Science*, 306(5693):117–120, 2004.

[263] Mark A Nakasone, Timothy A Lewis, Olivier Walker, Anita Thakur, Wissam Mansour, Carlos A Castañeda, Jennifer L Goeckeler-Fried, Frank Parlati, Tsui-Fen Chou, Ortal Hayat, et al. Structural basis for the inhibitory effects of ubistatins in the ubiquitin-proteasome pathway. *Structure*, 25(12):1839–1855, 2017.

[264] Yili Yang, Jirouta Kitagaki, Honghe Wang, De-Xing Hou, and Alan O Perantoni. Targeting the ubiquitin-proteasome system for cancer therapy. *Cancer science*, 100(1):24–28, 2009.

[265] R. C. Kane, P. F. Bross, A. T. Farrell, and R. Pazdur. Velcade: U.s. fda approval for the treatment of multiple myeloma progressing on prior therapy. *Oncologist*, 8(6):508–13, 2003.

[266] Paul G Richardson, Bart Barlogie, James Berenson, Seema Singhal, Sundar Jagannath, David Irwin, S Vincent Rajkumar, Gordan Srkalovic, Melissa Alsina, Raymond Alexanian,

et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. *New England Journal of Medicine*, 348(26):2609–2617, 2003.

[267] Min Shen, Sara Schmitt, Daniela Buac, and Q Ping Dou. Targeting the ubiquitin–proteasome system for cancer therapy. *Expert opinion on therapeutic targets*, 17(9):1091–1108, 2013.

[268] Alexei F Kisselev, Wouter A van der Linden, and Herman S Overkleeft. Proteasome inhibitors: An expanding army attacking a unique target. *Chemistry & biology*, 19(1):99–115, 2012.

[269] D Nix, C Pien, R Newman, T Madden, E Felix, J Adams, and P Elliott. Clinical development of a proteasome inhibitor, PS-341, for the treatment of cancer. In *Proc Am Soc Clin Oncol*, volume 20, page 86a, 2001.

[270] Ljudmila Borissenko and Michael Groll. 20S proteasome and its inhibitors: Crystallographic knowledge for drug development. *Chemical reviews*, 107(3):687–717, 2007.

[271] Anupama Pal, Matthew A Young, and Nicholas J Donato. Emerging potential of therapeutic targeting of ubiquitin-specific proteases in the treatment of cancer. *Cancer research*, 74(18):4955–4966, 2014.

[272] Shuqing Lü, Jianmin Yang, Zhilong Chen, Shenglan Gong, Hong Zhou, Xiaoqian Xu, and Jianmin Wang. Different mutants of PSMB5 confer varying bortezomib resistance in T lymphoblastic lymphoma/leukemia cells derived from the Jurkat cell line. *Experimental hematology*, 37(7):831–837, 2009.

[273] Ruud Oerlemans, Niels E Franke, Yehuda G Assaraf, Jacqueline Cloos, Ina van Zantwijk, Celia R Berkers, George L Scheffer, Kabir Debipersad, Katharina Vojtekova, Clara Lemos, et al. Molecular basis of bortezomib resistance: proteasome subunit $\beta 5$ (PSMB5) gene mutation and overexpression of PSMB5 protein. *Blood*, 112(6):2489–2499, 2008.

[274] NE Franke, D Niewerth, YG Assaraf, J Van Meerloo, K Vojtekova, CH Van Zantwijk, S Zweegman, ET Chan, CJ Kirk, DP Geerke, et al. Impaired bortezomib binding to mutant $\beta 5$ subunit of the proteasome is the underlying basis for bortezomib resistance in leukemia cells. *Leukemia*, 26(4):757, 2012.

[275] Dharminder Chauhan, Guilan Li, Reshma Shringarpure, Klaus Podar, Yasuyuki Ohtake, Teru Hideshima, and Kenneth C Anderson. Blockade of Hsp27 overcomes bortezomib/proteasome inhibitor PS-341 resistance in lymphoma cells. *Cancer research*, 63(19):6174–6177, 2003.

[276] Julian Adams and Michael Kauffman. Development of the proteasome inhibitor Velcade™ (Bortezomib). *Cancer investigation*, 22(2):304–311, 2004.

[277] K Martin Kortuem and A Keith Stewart. Carfilzomib. *Blood*, 121(6):893–897, 2013.

[278] Lihao Meng, Royce Mohan, Benjamin HB Kwok, Mikael Elofsson, Ny Sin, and Craig M Crews. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo anti-inflammatory activity. *Proceedings of the National Academy of Sciences*, 96(18):10403–10408, 1999.

[279] Amy M Ruschak, Malik Slassi, Lewis E Kay, and Aaron D Schimmer. Novel proteasome inhibitors to overcome bortezomib resistance. *Journal of the National Cancer Institute*, 103(13):1007–1017, 2011.

[280] Beverly A Teicher and Joseph E Tomaszewski. Proteasome inhibitors. *Biochemical pharmacology*, 96(1):1–9, 2015.

[281] David S Siegel, Thomas Martin, Michael Wang, Ravi Vij, Andrzej J Jakubowiak, Sagar Lonial, Suzanne Trudel, Vishal Kukreti, Nizar Bahlis, Melissa Alsina, et al. A phase 2 study of single-agent carfilzomib (PX-171-003-A1) in patients with relapsed and refractory multiple myeloma. *Blood*, pages blood–2012, 2012.

[282] Eva M Huber, Wolfgang Heinemeyer, and Michael Groll. Bortezomib-resistant mutant proteasomes: Structural and biochemical evaluation with carfilzomib and ONX 0914. *Structure*, 23(2):407–417, 2015.

[283] Ravi K Anchoori, Balasubramanyam Karanam, Shiwen Peng, Joshua W Wang, Rosie Jiang, Toshihiko Tanno, Robert Z Orlowski, William Matsui, Ming Zhao, Michelle A Rudek, et al. A bis-benzylidine piperidone targeting proteasome ubiquitin receptor RPN13/ADRM1 as a therapy for cancer. *Cancer cell*, 24(6):791–805, 2013.

[284] Y Song, A Ray, S Li, DS Das, YT Tai, RD Carrasco, D Chauhan, and KC Anderson. Targeting proteasome ubiquitin receptor Rpn13 in multiple myeloma. *Leukemia*, 30(9):1877, 2016.

[285] Darci J Trader, Scott Simanski, and Thomas Kodadek. A reversible and highly selective inhibitor of the proteasomal ubiquitin receptor rpn13 is toxic to multiple myeloma cells. *Journal of the American Chemical Society*, 137(19):6312–6319, 2015.

[286] Joseph J Sacco, Judy M Coulson, Michael J Clague, and Sylvie Urbé. Emerging roles of deubiquitinases in cancer-associated pathways. *IUBMB life*, 62(2):140–157, 2010.

[287] Xiangyun Chen, Jingjing Wu, Yitian Chen, Dongxia Ye, Hu Lei, Hanzhang Xu, Li Yang, Yingli Wu, and Wenli Gu. Ubiquitin-specific protease 14 regulates cell proliferation and apoptosis in oral squamous cell carcinoma. *The international journal of biochemistry & cell biology*, 79:350–359, 2016.

[288] X. Wang, W. Stafford, M. Mazurkiewicz, M. Fryknas, S. Brjnic, X. Zhang, J. Gullbo, R. Larsson, E. S. Arner, P. D'Arcy, and S. Linder. The 19s deubiquitinase inhibitor b-ap15 is enriched in cells and elicits rapid commitment to cell death. *Mol Pharmacol*, 85(6):932–45, 2014.

[289] Robin Didier, Aude Mallavialle, Rania Ben Jouira, Marie Angela Domdom, Mélanie Tichet, Patrick Auberger, Frédéric Luciano, Mickael Ohanna, Sophie Tartare-Deckert, and Marcel Deckert. Targeting the proteasome-associated deubiquitinating enzyme USP14 impairs melanoma cell survival and overcomes resistance to MAPK-targeting therapies. *Molecular cancer therapeutics*, 17(7):1416–1429, 2018.

[290] Kasyapa Chitta, Aneel Paulus, Sharoon Akhtar, Maja Kristin K Blake, Thomas R Caulfield, Anne J Novak, Stephen M Ansell, Pooja Advani, Sikander Ailawadhi, Taimur Sher, et al. Targeted inhibition of the deubiquitinating enzymes, USP14 and UCHL 5, induces proteotoxic stress and apoptosis in Waldenström macroglobulinaemia tumour cells. *British journal of haematology*, 169(3):377–390, 2015.

[291] A Paulus, S Akhtar, TR Caulfield, K Samuel, H Yousaf, Y Bashir, SM Paulus, D Tran, R Hudec, D Cogen, et al. Coinhibition of the deubiquitinating enzymes, USP14 and UCHL5, with VLX1570 is lethal to ibrutinib-or bortezomib-resistant Waldenström macroglobulinemia tumor cells. *Blood cancer journal*, 6(11):e492, 2016.

[292] Linda Lauinger, Jing Li, Anton Shostak, Ibrahim Avi Cemel, Nati Ha, Yaru Zhang, Philipp E Merkl, Simon Obermeyer, Nicolas Stankovic-Valentin, Tobias Schafmeier, et al. Thiolutin is a zinc chelator that inhibits the rpn11 and other jamm metalloproteases. *Nature chemical biology*, 13(7):709, 2017.

[293] Dingding Shi and Steven R Grossman. Ubiquitin becomes ubiquitous in cancer: Emerging roles of ubiquitin ligases and deubiquitinases in tumorigenesis and as therapeutic targets. *Cancer biology & therapy*, 10(8):737–747, 2010.

[294] Karthik Selvaraju, Magdalena Mazurkiewicz, Xin Wang, Joachim Gullbo, Stig Linder, and Pádraig D'Arcy. Inhibition of proteasome deubiquitinase activity: A strategy to overcome resistance to conventional proteasome inhibitors? *Drug resistance updates*, 21:20–29, 2015.

[295] Kathleen Coughlin, Ravi Anchoori, Yoshie Iizuka, Joyce Meints, Lauren MacNeill, Rachel Isaksson Vogel, Robert Z Orlowski, Michael K Lee, Richard BS Roden, and Martina Bazzaro. Small-molecule ra-9 inhibits proteasome-associated dubs and ovarian cancer in vitro and in vivo via exacerbating unfolded protein responses. *Clinical cancer research*, 20(12):3174–3186, 2014.

[296] JE Mullally and FA Fitzpatrick. Pharmacophore model for novel inhibitors of ubiquitin isopeptidases that induce p53-independent cell death. *Molecular Pharmacology*, 62(2):351–358, 2002.

[297] Shishir Shishodia, Gautam Sethi, and Bharat B Aggarwal. Curcumin: Getting back to the roots. *Annals of the New York Academy of Sciences*, 1056(1):206–217, 2005.

[298] Bharat B Aggarwal, Chitra Sundaram, Nikita Malani, and Haruyo Ichikawa. Curcumin: the Indian solid gold. In *The molecular targets and therapeutic uses of curcumin in health and disease*, pages 1–75. Springer, 2007.

[299] Pandima Devi Kasi, Rajavel Tamilselvam, Krystyna Skalicka-Woźniak, Seyed Fazel Nabavi, Maria Daglia, Anupam Bishayee, Hamidreza Pazoki-toroudi, and Seyed Mohammad Nabavi. Molecular targets of curcumin for cancer therapy: an updated review. *Tumor Biology*, 37(10):13017–13028, 2016.

[300] Preetha Anand, Ajaikumar B Kunnumakkara, Robert A Newman, and Bharat B Aggarwal. Bioavailability of curcumin: Problems and promises. *Molecular pharmaceutics*, 4(6):807–818, 2007.

[301] Simona D'Aguanno, Igea D'Agnano, Michele De Canio, Claudia Rossi, Sergio Bernardini, Giorgio Federici, and Andrea Urbani. Shotgun proteomics and network analysis of neuroblastoma cell lines treated with curcumin. *Molecular Biosystems*, 8(4):1068–1077, 2012.

[302] Vesna Milacic, Sanjeev Banerjee, Kristin R Landis-Piwowar, Fazlul H Sarkar, Adhip PN Majumdar, and Q Ping Dou. Curcumin inhibits the proteasome activity in human colon cancer cells in vitro and in vivo. *Cancer research*, 68(18):7283–7292, 2008.

[303] Han-Zhong Zhang, Shailaja Kasibhatla, Yan Wang, John Herich, John Guastella, Ben Tseng, John Drewe, and Sui Xiong Cai. Discovery, characterization and SAR of gambogic acid as a potent apoptosis inducer by a HTS assay. *Bioorganic & medicinal chemistry*, 12(2):309–317, 2004.

[304] Teresita Reiner, Ricardo Parrondo, Alicia de las Pozas, Deanna Palenzuela, and Carlos Perez-Stable. Betulinic acid selectively increases protein degradation and enhances prostate cancer-specific apoptosis: possible role for inhibition of deubiquitinase activity. *PloS one*, 8(2):e56234, 2013.

[305] RA Sharma, AJ Gescher, and WP Steward. Curcumin: the story so far. *European journal of cancer*, 41(13):1955–1968, 2005.

[306] Binhua Zhou, Yinglin Zuo, Baojian Li, Hao Wang, Hao Liu, Xianfeng Wang, Xu Qiu, Yun Hu, Shijun Wen, Jun Du, et al. Deubiquitinase inhibition of 19S regulatory particles by 4-arylidene curcumin analog AC17 causes NF-κB inhibition and p53 reactivation in human lung cancer cells. *Molecular cancer therapeutics*, 2013.

[307] Xin Yue, Yinglin Zuo, Hongpeng Ke, Jiaming Luo, Lanlan Lou, Wenjing Qin, Youqiao Wang, Ziyi Liu, Daoyuan Chen, Haixia Sun, et al. Identification of 4-arylidene curcumin analogues as novel proteasome inhibitors for potential anticancer agents targeting 19S regulatory particle associated deubiquitinase. *Biochemical pharmacology*, 137:29–50, 2017.

[308] Vaibhav Kapuria, Luke F Peterson, Dexing Fang, William G Bornmann, Moshe Talpaz, and Nicholas J Donato. Deubiquitinase inhibition by small-molecule WP1130 triggers aggresome formation and tumor cell apoptosis. *Cancer Research*, 70(22):9265–76, 2010.

[309] Jeffrey W Perry, Mohammad Ahmed, Kyeong-Ok Chang, Nicholas J Donato, Hollis D Showalter, and Christiane E Wobus. Antiviral activity of a small molecule deubiquitinase inhibitor occurs via induction of the unfolded protein response. *PLoS pathogens*, 8(7):e1002783, 2012.

[310] Geoffrey A Bartholomeusz, Moshe Talpaz, Vaibhav Kapuria, Ling Yuan Kong, Shimei Wang, Zeev Estrov, Waldemar Priebe, Ji Wu, and Nicholas J Donato. Activation of a novel Bcr/Abl destruction pathway by WP1130 induces apoptosis of chronic myelogenous leukemia cells. *Blood*, 109(8):3470–3478, 2007.

[311] Peifen Fu, Feiya Du, Yu Liu, Minya Yao, Shufeng Zhang, Xiaoxiao Zheng, and Shusen Zheng. WP1130 increases cisplatin sensitivity through inhibition of USP9x in estrogen receptor-negative breast cancer cells. *American journal of translational research*, 9(4):1783, 2017.

[312] Ningning Liu, Xiaofen Li, Hongbiao Huang, Chong Zhao, Siyan Liao, Changshan Yang, Shouting Liu, Wenbin Song, Xiaoyu Lu, Xiaoying Lan, et al. Clinically used antirheumatic agent auranofin is a proteasomal deubiquitinase inhibitor and inhibits tumor growth. *Oncotarget*, 5(14):5453, 2014.

[313] Ningning Liu, Hongbiao Huang, Q Ping Dou, and Jinbao Liu. Inhibition of 19S proteasome-associated deubiquitinases by metal-containing compounds. *Oncoscience*, 2(5):457, 2015.

[314] Ningning Liu, Chunjiao Liu, Xiaofen Li, Siyan Liao, Wenbin Song, Changshan Yang, Chong Zhao, Hongbiao Huang, Lixia Guan, Peiquan Zhang, et al. A novel proteasome inhibitor suppresses tumor growth via targeting both 19S proteasome deubiquitinases and 20S proteolytic peptidases. *Scientific reports*, 4:5240, 2014.

[315] Sonia Ciotti, Riccardo Sgarra, Andrea Sgorbissa, Carlotta Penzo, Andrea Tomasella, Federico Casarsa, Fabio Benedetti, Federico Berti, Guidalberto Manfioletti, and Claudio Brancolini. The binding landscape of a partially-selective isopeptidase inhibitor with potent pro-death activity, based on the bis (arylidene) cyclohexanone scaffold. *Cell death & disease*, 9(2):184, 2018.

[316] Xiaonan Zhang, Karthik Selvaraju, Amir Ata Saei, Padraig D'Arcy, Roman A Zubarev, Elias SJ Arnér, and Stig Linder. Repurposing of auranofin: thioredoxin reductase remains a primary target of the drug. *Biochimie*, 2019.

[317] Dorthe Groth Petersen, Ingela Dahllof, and Lars Peter Nielsen. Effects of zinc pyrithione and copper pyrithione on microbial community function and structure in sediments. *Environmental Toxicology and Chemistry*, 23(4):921–928, 2004.

[318] Chong Zhao, Xin Chen, Changshan Yang, Dan Zang, Xiaoying Lan, Siyan Liao, Peiquan Zhang, Jinjie Wu, Xiaofen Li, Ningning Liu, et al. Repurposing an antidandruff agent to treating cancer: zinc pyrithione inhibits tumor growth via targeting proteasome-associated deubiquitinases. *Oncotarget*, 8(8):13942, 2017.

[319] Xin Chen, Jinjie Wu, Qianqian Yang, Xiaolan Zhang, Peiquan Zhang, Siyan Liao, Zhimin He, Xuejun Wang, Chong Zhao, and Jinbao Liu. Cadmium pyrithione suppresses tumor growth in vitro and in vivo through inhibition of proteasomal deubiquitinase. *BioMetals*, 31(1):29–43, 2018.

[320] Emanuela Aleo, Clare J Henderson, Alessandra Fontanini, Barbara Solazzo, and Claudio Brancolini. Identification of new compounds that trigger apoptosome-independent caspase activation and apoptosis. *Cancer research*, 66(18):9235–9244, 2006.

[321] Ulma Cersosimo, Andrea Sgorbissa, Carmen Foti, Sara Drioli, Rosario Angelica, Andrea Tomasella, Raffaella Picco, Marta Stefania Semrau, Paola Storici, Fabio Benedetti, et al. Synthesis, characterization, and optimization for in vivo delivery of a nonselective isopeptidase inhibitor as new antineoplastic agent. *Journal of medicinal chemistry*, 58(4):1691–1704, 2015.

[322] Caroline Haglund, Chitralekha Mohanty, Mårten Fryknäs, Padraig D'Arcy, Rolf Larsson, Stig Linder, and Linda Rickardson. Identification of an inhibitor of the ubiquitin–proteasome system that induces accumulation of polyubiquitinated proteins in the absence of blocking of proteasome function. *MedChemComm*, 5(3):376, 2014.

[323] Ze Tian, Padraig D'Arcy, Xin Wang, Arghya Ray, Yu-Tzu Tai, Yiguo Hu, Ruben D Carrasco, Paul Richardson, Stig Linder, Dharminder Chauhan, et al. A novel small molecule inhibitor of deubiquitylating enzyme USP14 and UCHL5 induces apoptosis in multiple myeloma and overcomes bortezomib resistance. *Blood*, 123(5):706–716, 2014.

[324] Magdalena Mazurkiewicz, Ellin-Kristina Hillert, Xin Wang, Paola Pellegrini, Maria Hägg Olofsson, Karthik Selvaraju, Padraig D'Arcy, and Stig Linder. Acute lymphoblastic leukemia cells are sensitive to disturbances in protein homeostasis induced by proteasome deubiquitinase inhibition. *Oncotarget*, 8(13):21115, 2017.

[325] Slavica Brnjic, Magdalena Mazurkiewicz, Mårten Fryknäs, Chao Sun, Xiaonan Zhang, Rolf Larsson, Pádraig D'Arcy, and Stig Linder. Induction of tumor cell apoptosis by a proteasome deubiquitinase inhibitor is associated with oxidative stress. *Antioxidants & redox signaling*, 21(17):2271–2285, 2014.

[326] Xin Wang, Pádraig D'arcy, Thomas R Caulfield, Aneel Paulus, Kasyapa Chitta, Chitralekha Mohanty, Joachim Gullbo, Asher Chanan-Khan, and Stig Linder. Synthesis and evaluation of derivatives of the proteasome deubiquitinase inhibitor b-AP15. *Chemical biology & drug design*, 86(5):1036–1048, 2015.

[327] Stig T. Linder, Padraig D'Arcy, and Xiaonan Zhang. Abstract 3207: Targetting proteasome function by inhibition of the proteasome deubiquitinase usp14. *Cancer Research*, 77(13 Supplement):3207–3207, 2017.

[328] William H Chappell, Linda S Steelman, Jacquelyn M Long, Ruth C Kempf, Stephen L Abrams, Richard A Franklin, Jörg Bäsecke, Franca Stivala, Marco Donia, Paolo Fagone, et al. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. *Oncotarget*, 2(3):135, 2011.

[329] Jürgen Besemer, Hanna Harant, Shirley Wang, Berndt Oberhauser, Katharina Marquardt, Carolyn A Foster, Erwin P Schreiner, Jan E de Vries, Christiane Dascher-Nadel, and Ivan JD Lindley. Selective inhibition of cotranslational translocation of vascular cell adhesion molecule 1. *Nature*, 436(7048):290, 2005.

[330] Derek Narendra, Atsushi Tanaka, Der-Fen Suen, and Richard J Youle. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *The Journal of cell biology*, 183(5):795–803, 2008.

[331] Noor Hasima and Bharat B Aggarwal. Targeting proteasomal pathways by dietary curcumin for cancer prevention and treatment. *Current medicinal chemistry*, 21(14):1583–1594, 2014.

[332] Malin Jarvius, Mårten Fryknäs, Pädraig D'Arcy, Chao Sun, Linda Rickardson, Joachim Gullbo, Caroline Haglund, Peter Nygren, Stig Linder, and Rolf Larsson. Piperlongumine induces inhibition of the ubiquitin–proteasome system in cancer cells. *Biochemical and biophysical research communications*, 431(2):117–123, 2013.

[333] Xiaofen Li, Shouting Liu, Hongbiao Huang, Ningning Liu, Chong Zhao, Siyan Liao, Changshan Yang, Yurong Liu, Canguo Zhao, Shujue Li, et al. Gambogic acid is a tissue-specific proteasome inhibitor in vitro and in vivo. *Cell reports*, 3(1):211–222, 2013.

[334] Emily J Noonan, Robert F Place, Charles Giardina, and Lawrence E Hightower. Hsp70B regulation and function. *Cell stress & chaperones*, 12(4):393, 2007.

[335] Leo E Otterbein and Augustine MK Choi. Heme oxygenase: colors of defense against cellular stress. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 279(6):L1029–L1037, 2000.

[336] Leo E Otterbein, Miguel P Soares, Kenichiro Yamashita, and Fritz H Bach. Heme oxygenase-1: unleashing the protective properties of heme. *Trends in immunology*, 24(8):449–455, 2003.

[337] Fumihiko Urano, XiaoZhong Wang, Anne Bertolotti, Yuhong Zhang, Peter Chung, Heather P Harding, and David Ron. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science*, 287(5453):664–666, 2000.

[338] Dhifaf Sarhan, Erik Wennerberg, Padraig D'Arcy, Deepthy Gurajada, Stig Linder, and Andreas Lundqvist. A novel inhibitor of proteasome deubiquitinating activity renders tumor cells sensitive to trail-mediated apoptosis by natural killer cells and t cells. *Cancer Immunology, Immunotherapy*, 62(8):1359–1368, 2013.

[339] Noriyuki Matsuda, Shigeto Sato, Kahori Shiba, Kei Okatsu, Keiko Saisho, Clement A Gautier, Yu-shin Sou, Shinji Saiki, Sumihiro Kawajiri, Fumiaki Sato, et al. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *The Journal of cell biology*, 189(2):211–221, 2010.

[340] Jonathan B Baell. Feeling nature's PAINS: natural products, natural product drugs, and pan assay interference compounds (PAINS). *Journal of natural products*, 79(3):616–628, 2016.

[341] Jonathan B Baell and Georgina A Holloway. New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *Journal of medicinal chemistry*, 53(7):2719–2740, 2010.

[342] Ashley Mooneyham and Martina Bazzaro. Targeting deubiquitinating enzymes and autophagy in cancer. In *Cancer Gene Networks*, pages 49–59. Springer, 2017.

[343] R. W. King. Enhancing proteasome activity by inhibiting the proteasome-associated deubiquitinating enzyme USP14. *Abstracts of Papers of the American Chemical Society*, 241, 2011.

[344] Magdalena J Kiprowska, Anna Stepanova, Dustin R Todaro, Alexander Galkin, Arthur Haas, Scott M Wilson, and Maria E Figueiredo-Pereira. Neurotoxic mechanisms by which the USP14 inhibitor IU1 depletes ubiquitinated proteins and Tau in rat cerebral cortical neurons: Relevance to Alzheimer's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1863(6):1157–1170, 2017.

[345] Eunkyoung Kim, Seoyoung Park, Jung Hoon Lee, Ji Young Mun, Won Hoon Choi, Yejin Yun, Jeeyoung Lee, Ji Hyeon Kim, Min-Ji Kang, and Min Jae Lee. Dual function of USP14 deubiquitinase in cellular proteasomal activity and autophagic flux. *Cell reports*, 24(3):732–743, 2018.

[346] U. Ponnappan, S. Ponnappan, H. Ovaa, S. Cane, D. Sullivan, and M. Palmieri. Increased activity of proteasome associated deubiquitinating enzyme, USP14, accompanies lowered proteasomal proteolysis in primary human T lymphocytes during aging. *Journal of Immunology*, 186, 2011.

[347] Yiwei Wang, Yuxuan Jiang, Shan Ding, Jiawang Li, Ningjing Song, Yujing Ren, Danning Hong, Cai Wu, Bin Li, Feng Wang, et al. Small molecule inhibitors reveal allosteric regulation of USP14 via steric blockade. *Cell research*, 28(12):1186, 2018.

[348] Daniel Ortuno, Holly J Carlisle, and Silke Miller. Does inactivation of USP14 enhance degradation of proteasomal substrates that are associated with neurodegenerative diseases? *F1000Research*, 5, 2016.

[349] Hyoung Tae Kim and Alfred L Goldberg. UBL domain of Usp14 and other proteins stimulates proteasome activities and protein degradation in cells. *Proceedings of the National Academy of Sciences*, 115(50):E11642–E11650, 2018.

[350] Adi Guterman and Michael H Glickman. Complementary roles for Rpn11 and Ubp6 in deubiquitination and proteolysis by the proteasome. *Journal of Biological Chemistry*, 279(3):1729–1738, 2004.

[351] Alise Hyrskyluoto, Céline Bruelle, Sofia H Lundh, Hai Thi Do, Jenny Kivinen, Elisabeth Rappou, Sami Reijonen, Tuure Waltimo, Åsa Petersén, Dan Lindholm, et al. Ubiquitin-specific protease-14 reduces cellular aggregates and protects against mutant huntingtin-induced cell degeneration: involvement of the proteasome and ER stress-activated kinase IRE1α. *Human molecular genetics*, 23(22):5928–5939, 2014.

[352] Scott M Wilson, Bula Bhattacharyya, Rivka A Rachel, Vincenzo Coppola, Lino Tessarollo, Deborah B Householder, Colin F Fletcher, Richard J Miller, Neal G Copeland, and Nancy A Jenkins. Synaptic defects in ataxia mice result from a mutation in USP14, encoding a ubiquitin-specific protease. *Nature genetics*, 32(3):420, 2002.

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